PATENT
Customer No. 76,392
Attorney Docket No. SP-01-US-DIV-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:	)	
	)	
Campbell et al.	)	Group Art Unit: 1632
	)	
Application No.: 09/225,233	)	Examiner: D. Crouch
	)	
Filed: January 4, 1999	)	Confirmation No.: 2711
	)	
For: QUIESCENT CELL POPULATIONS FOR NUCLEAR TRANSFER	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

APPEAL BRIEF UNDER 37 C.F.R § 41.37

Pursuant to 37 C.F.R § 41.37, Appellant submits this Appeal Brief in response to the November 10, 2008, final rejection of claims 155-159 and 164. A Notice of Appeal was filed on May 8, 2009. This Appeal Brief is accompanied by a payment of the fee of $540.00 as required under 37 C.F.R § 41.20(b)(2) and a petition and fee for a two month extension of time.

STATEMENT OF THE REAL PARTY IN INTEREST

The real parties in interest are Start Licensing, Inc., of Austin, Texas, a wholly-owned subsidiary of ViaGen of Austin, Texas, by virtue of a licensing agreement, Geron Corporation of Menlo Park, California, by virtue of a licensing agreement; Exeter Life Sciences, Inc., of Phoenix, Arizona, by virtue of a licensing agreement, and Roslin Institute (Edinburgh) of Midlothian, Great Britain, by virtue of an assignment from the inventors, an assignment from the Biotechnology & Biological Sciences Research Council, which was an assignee, and an assignment from Department for Environment, Food & Rural Affairs, London, Great Britain, having taken over the governmental functions of the Minister of Agriculture, Fisheries & Food, which was an assignee.

STATEMENT OF RELATED CASES

In accordance with 37 C.F.R. § 41.37(c)(1)(ii), Appellant advises the Board of Patent Appeals and Interferences of the following prior or pending appeals, interferences, or judicial proceedings which may be related to, directly affect, or would be directly affected by or have a bearing on the Board's decision in the instant appeal: a prior appeal filed in U.S. application 09/658,862 on June 16, 2006, and a prior appeal filed in the instant case on June 16, 2006.

JURISDICTIONAL STATEMENT

The Board has jurisdiction under 35 U.S.C. § 134(a). The Examiner mailed a rejection on November 10, 2008, setting a three-month shortened statutory period for response. The claims had been previously finally rejected in an Office Action dated August 17, 2005. The time for responding to the rejection expired on May 10, 2009. 37 C.F.R § 1.134. A notice of appeal and a petition for a three month extension of time were filed on May 8, 2009. The time for filing an appeal brief is two months after the filing of a notice of appeal. Bd.R. 41.37(c). The time for filing an appeal brief expired on July 8, 2009. The appeal brief is being filed on September 8, 2009, with a petition and fee for a two month extension of time.

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STATUS OF AMENDMENTS

No amendments have been filed subsequent to the rejection on November 10, 2008. Two Terminal Disclaimers were filed on May 8, 2009, in response to double patenting rejections over a number of issued U.S. patents. According to the USPTO PAIR system, these terminal disclaimers were approved. Thus, Appellant assumes that these rejections are moot.

GROUNDS OF REJECTION TO BE REVIEWED

Appellant requests review of the rejection by the Examiner of claims 155-159 and 164 under 35 U.S.C. § 101 as allegedly being directed to nonstatutory subject matter. Appellant also requests review of the rejection by the Examiner of claims 155-159 and 164 under 35 U.S.C. § 102(b) or 35 U.S.C. § 103(a) for allegedly being anticipated by or obvious over several publications that teach embryo cloned cattle (Sims et al., 1993), sheep (McLaughlin et al., 1990), pigs (Prather et al., 1989), and goats (Yong et al., 1991). Appellant further requests review of the rejection by the Examiner of claims 155-159 and 164 under 35 U.S.C. § 102(b) or 35 U.S.C. § 103(a) for allegedly being anticipated by or obvious over several publications that teach ovum fertilized by sperm in cattle (Zinn, 1993), sheep (Aldrich et al., 1990), pigs (Matte et al., 1993), and goats (Ortega-Reyes, 1993).

STATEMENT OF FACTS

1. In a prior decision in this application dated January 30, 2008, the Board of Patent Appeals and Interferences admitted that Appellant's claimed clone "will not be an exact copy of the 'parent.'" January 30, 2008, Decision ("Decision") at 14.

2. The Board conceded that environmental factors will result in physical differences between the clone and its parent. *Id.*

3. The Board further admitted that Appellant's claimed clone and its parent will occupy a different space and time and will have phenotypic differences. *Id.* at 15.

4. The Examiner has also admitted that the clone and the donor animal will have differences. November 10, 2008, Office Action at 10.

5. Claims 155-159 and 164 do not recite any process steps. Claims 155-159 and 164.

6. Rather, claims 155-159 and 164 require a ***clone*** of a pre-existing, non-embryonic, donor mammal. Claims 155-159 and 164.

7. Appellant explained in the specification that a clone is not strictly identical to its parent. Specification at 19, lines 7-21.

8. Cloned offspring may vary phenotypically due to environment. Prather et al., 1990, at 10, col. 2, 3rd full ¶.

9. In addition, differences in mitochondrial DNA (from the oocyte) may

contribute to differences between a clone and its parent. *Id.*, at 10, col. 1-2, bridging ¶.

10. Moreover, environmental factors, such a uterine environment, generate differences that prevent a clone and its parent from being phenotypically identical. *Id.* at 10-11.

11. Differences in phenotype may be manifested in many ways. For example, a clone and its parent may have phenotypic differences in color patterns caused by a different migration of melanocytes during development. Prather et al., 1990, at 10, 4th full ¶, Wells et al., 1999, at 1003, col. 2, 2nd full ¶.

12. The iris of every eye is unique. U.S. Patent 4,641,349 at col. 4, lines 37-58.

13. Consequently, a clone will have a different pigmentation of the iris as compared to its parent. *Id.*

14. Thus, a clone that contains the same set of chromosomes as a single parental mammal can be distinguished from the parental mammal due to these environmental influences. February 26, 2003, Declaration of Dr. David Wells at ¶35.

15. Also, the cloned mammal will have behavioral differences from the parental mammal. *Id.*

16. Since Appellant's claims require that the clone is of a pre-existing mammal, the cloned mammal will always be of a younger age than the parental mammal

since the parental mammal must exist before the clone can exist. *Id.* at ¶35.

17. As a result, the clone and the parental mammal cannot be ***identical***. *Id.*

18. In its previous decision in this application, the Board found that: "It does not appear on this record that Campbell or the art intended to use the term 'clone' in the 'true sense of the meaning,' which we understand to be an exact copy. January 30, 2008, Decision at 27, footnote 18.

19. A mammal cloned by somatic cell nuclear transfer will contain the same set of chromosomes as a single parental mammal. February 26, 2003, Declaration of Dr. David Wells at ¶34.

20. A mammal cloned by somatic cell nuclear transfer is unlike any mammal produced by a process involving sexual reproduction, including a mammal produced by nuclear transfer from an embryonic cell. *Id.*

21. The set of chromosomes of a mammal cloned by somatic cell nuclear transfer is obtained from a single parental mammal. *Id.*

22. The set of chromosomes from any mammal produced by a process involving sexual reproduction comes from 2 parental mammals, one male and one female. *Id.*

23. This feature allows the cloned mammal to preserve the genetic information of the parental mammal without dilution. *Id.*

24. In its previous decision in this application, the Board found that: "A comparison of the chromosomal DNA of the claimed clones with that of the donor

mammal would be expected to show virtual identity." January 30, 2008, Decision at 27, second ¶.

25. In its previous decision in this application, the Board further found that: "In contrast, a comparison of the DNA of sexually reproduced horses and rats of the references with that of either one of the parents would be expected to show only 50% identity." *Id.*

26. In its previous Decision in this application, the Board phrased the critical question of anticipation/obviousness as: "does the identity of the nuclear genetic code make a patentable difference?" *Id.* at 27, third ¶.

27. In its previous Decision in this application, the Board answered this question in the affirmative and concluded that Appellant's claims to horses and rats were not anticipated or obvious over other previously cited references, stating: "The Examiner has not relied on any evidence that a horse or rat having the same nuclear genetic code as a previously existing horse or rat existed or was enabled prior to Campbell's disclosure." *Id.* at 27-28.

28. Appellant's clone is not made by nature. Declaration of Irina A. Polejaeva, Ph.D, Under 37 C.F.R. § 1.132 at ¶94.

29. Appellant's clone can only be made by human intervention. *Id.* at ¶95.

30. Appellant's clone is not an exact copy of its donor mammal. *Id.* at ¶96.

31. Environmental factors would generate differences between Appellant's

clone and its donor mammal. *Id.* at ¶97.

32. Appellant's clone occupies different space and time than its donor mammal, and is a time-delayed, inexact copy of its donor mammal. *Id.* at ¶99.

33. The ability of Appellant's clone to exist at a time later than its donor mammal, but have the same genetic complement, is a markedly different characteristic from any mammal found in nature. *Id.* at ¶100.

34. No mammal found in nature is a time-delayed copy of either of its parent. *Id.* at ¶101.

35. Appellant's clone provides an alternative, time-delayed source of nuclear genomic material of its donor mammal. *Id.* at ¶102.

36. This feature of Appellant's clone does not depend on the continued existence of the donor mammal. *Id.* at ¶103.

37. Appellant's clone can provide an alternative source of nuclear genomic material of its donor mammal, even if the donor mammal is dead. *Id.* at ¶104.

38. Appellant's clone allows the preservation of the genomic material of a particular mammal beyond the normal lifespan of that mammal. *Id.* at ¶105.

39. Normally, when a mammal dies, its particular genomic composition is lost. *Id.* at ¶106.

40. Its progeny only contain one-half of each of its two parents' genomic material. *Id.* at ¶106.

41. The genomic material of one parent is inextricably scrambled together with the other parent's genomic material to create the progeny. *Id.* at ¶106.

42. Appellant's clone avoids this permanent loss of a particular genomic composition. *Id.* at ¶107.

43. Appellant's clone provides the potential for the continuation of a particular genomic composition in a way that never occurs in nature. *Id.* at ¶108.

44. This cannot be considered a trivial difference from a clone's donor mammal, which does not have this potential. *Id.* at ¶109.

45. Appellant's clone requires two animals, namely, a pre-existing, non-embryonic, donor mammal and a clone of that donor mammal. *Id.* at ¶110.

46. Nature never makes such a pair of mammals. *Id.* at ¶111.

47. Prior to the birth of Dolly, as reported in Wilmut et al., Viable offspring derived from fetal and adult mammalian cells, Nature 385:810-13 (1997), the skilled artisan would have expected that the generation of a live-born clone of a pre-existing, non-embryonic, donor mammal was not possible. *Id.* at ¶167.

48. Prior to the birth of Dolly, the skilled artisan would not have been expected that is was possible to generate a cloned mammal using non-embryonic nuclear donor cells. *Id.* at ¶168.

49. Sims et al. (1993) reports the production of calves by nuclear transfer using cultured inner cell mass cells. Declaration of Irina A. Polejaeva, Ph.D, under 37

C.F.R. § 1.132 at ¶114.

50. In Sims et al. (1993), the inner cell mass cells were generated by performing immunosurgery on in vitro cultured embryos. *Id.* at ¶115.

51. In Sims et al. (1993), the in vitro cultured embryos were generated by in vitro fertilizing oocytes with sperm. *Id.* at ¶116.

52. Thus, sexual reproduction was used to generate embryos used to generate the cells for the nuclear transfer procedures of Sims et al. (1993). *Id.* at ¶117.

53. The calves of Sims et al. (1993) were derived using cells derived from embryos as the nuclear donor cells. *Id.* at ¶118.

54. These embryos were destroyed during the procedure used to make the donor cells for nuclear transfer. *Id.* at ¶119.

55. McLaughlin et al. (1990) reports the production of lambs by nuclear transfer using cells from embryos at the 8- to 16-cell stage. *Id.* at ¶121.

56. In McLaughlin et al. (1990), the embryos were generated by artificial insemination with sperm. *Id.* at ¶122.

57. Thus, sexual reproduction was used to generate the embryos used to generate the cells for the nuclear transfer procedures of McLaughlin et al. (1990). *Id.* at ¶123.

58. The lambs of McLaughlin et al. (1990) were all derived using cells derived from embryos as the nuclear donor. *Id.* at ¶124.

59. These embryos were destroyed during the procedure used to make the cells for nuclear transfer. *Id.* at ¶125.

60. Prather et al. (1989) reports the production of a pig by nuclear transfer using a cell from an embryo at the 4-cell stage. *Id.* at ¶127.

61. In Prather et al. (1989), the embryo was generated by breeding pigs. *Id.* at ¶128.

62. Thus, sexual reproduction was used to generate embryos used to generate the cells for the nuclear transfer procedures of Prather et al. (1989). *Id.* at ¶129.

63. The pig of Prather et al. (1989) was derived using a cell derived from an embryo as the nuclear donor. *Id.* at ¶130.

64. The embryo was destroyed during the procedure used to make the cells for nuclear transfer. *Id.* at ¶131.

65. Yong et al. (1991) reports the production of goats by nuclear transfer using cells from embryos at the 4- to 32-cell cell stage. *Id.* at ¶133.

66. In Yong et al. (1991), the embryos were generated from pregnant goats. *Id.* at ¶134.

67. Thus, sexual reproduction was used to generate the embryos used to generate the cells for the nuclear transfer procedures of Yong et al. (1991). *Id.* at ¶135.

68. The goats of Yong et al. (1991) were all derived using cells derived from

embryos as the nuclear donor. *Id.* at ¶136.

69. These embryos were destroyed during the procedure used to make the cells for nuclear transfer. *Id.* at ¶137.

ARGUMENT

A. Rejections under 35 U.S.C. § 101

The Examiner rejected claims 155-159 and 164 under 35 U.S.C. § 101 for allegedly being directed to non-statutory subject matter. It is the Examiner's position that Appellant's claimed mammals are indistinguishable from mammals found in nature. (November 10, 2008, Office Action at 7.)

Appellant previously pointed out where the Examiner erred in the Amendment filed March 30, 2008, on pages 3-9. The Examiner has misconstrued Appellant's claims and performed an improper analysis under 35 U.S.C. § 101. Under a proper legal analysis, Appellant's claims are patentable.

Appellant's claims are not product-by-process claims

Although Appellant's claims do not recite any method for making the claimed clone, the Examiner and the Board contend that claims 155-159 and 164 are product-by-process claims. (November 10, 2008, Office Action at 13, first ¶; Decision at 28, first full ¶.) However, Appellant's claims are not product-by-process claims.

Although a clone can only be made by "cloning" the parent, a similar

argument can be made of any product requiring human intervention. A composition of matter can only be made by bringing together the materials that make up the composition. An apparatus can only be made by assembling its components. A manufacture can only be made by manufacturing it. Thus, simply because a clone requires cloning does not change Appellant's claims into product-by-process claims. The term "clone" should be viewed as a structural limitation.

Appellant's claims 155-159 and 164 do not recite any process steps. (Fact 5.) Rather, these claims require a ***clone*** of a pre-existing, non-embryonic, donor mammal. (Fact 6.) The clone can be made by any method. The limitation "clone" is a structural limitation. That is, the clone must be a structural "copy" of the parent. It must have the same genetic complement as its donor animal. (Facts 19 and 21.)

When possible, claims are construed by the court as having structural limitations instead of process limitations. *See, e.g., In re Garnero*, 412 F.2d 276, 278-279 (CCPA 1969) ("However, it seems to us that the recitation of the particles as 'interbonded one to another by interfusion between the surfaces of the perlite particles' is as capable of being construed as a structural limitation as 'intermixed,' 'ground in place,' 'press fitted,' 'etched,' and 'welded,' all of which at one time or another have been separately held capable of construction as structural, rather than process, limitations."); *see also Hazani v. United States Int'l Trade Comm'n*, 126

F.3d 1473, 1479 (Fed. Cir. 1997) ("We agree with the respondents, however, that those claims are best characterized as pure product claims, since the "chemically engraved" limitation, read in context, describes the product more by its structure than by the process used to obtain it.").

Even words of limitation that can connote with equal force a structural characteristic of the product or a process of manufacture are commonly and by default interpreted in their structural sense, unless the patentee has demonstrated otherwise. *3M Innovative Properties Co. v. Avery Dennison Corp.*, 350 F.3d 1365, 1371 (Fed. Cir. 2004). Consequently, the term "clone" in Appellant's claims should be construed as a structural limitation.

The Examiner contends that: "[t]he phrase 'live born clone of a . . . mammal' imbues the method by which the clone was made, nuclear transfer." (Office Action at 13, first ¶.) However, even if a process limitation appears in a claim, this does not convert it to a product by process claim. *Fromson v. Advance Offset Plate, Inc.*, 720 F.2d 1565, 1570 (Fed. Cir. 1983). For example, the phrase "condensation product of formaldehyde with a salt of . . . " does not convert a product claim to a "product-by-process" claim. *In re Steppan*, 394 F.2d 1013, 1017 (CCPA 1967). Similarly, the phrase, "purified from mammalian cells grown in culture" was held not to be a product-by-process limitation. *Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1329 (Fed. Cir. 2003). Likewise,

the term "clone" in Appellant's claims does not convert them into product-by-process claims.

In view of legal precedent, Appellant's claims 155-159 and 164 are properly construed as product claims, and not product-by-process claims.

Appellant's claims fulfill the requirements of 35 U.S.C. § 101

As held by the Supreme Court in *Diamond v. Chakrabarty*, statutory subject matter includes "anything under the sun that is made by man." 447 U. S. 303, 308 (1980). The relevant distinction between non-statutory and statutory subject matter is between products of nature, whether living or not, and human-made inventions. *Diamond v. Chakrabarty*, 447 U. S. 303 at 313. Appellant's claimed clones are statutory subject matter since they must be made by man.

Appellant's clones are never found in nature. (Fact 28.) Since mammals do not naturally reproduce by cloning, Appellant's clone can only be made by human intervention. (Fact 29.) Thus, a cloned mammal encompassed by Appellant's claims is a non-naturally occurring product of human ingenuity. Consequently, Appellant is not claiming a product of nature, but one that is made by man. Since clones of mammals are not products of nature, but are human-made inventions, the requirements of 35 U.S.C. § 101 are fulfilled by Appellant's claims.

The Examiner's allegation that there are no differences between mammals produced by mating, IVF, or cloning (November 10, 2008, Office Action at 7-8,

bridging ¶) is in error. Only a mammal produced by cloning has the same genetic complement as its parent. (Fact 19.) Mammals produced by IVF or mating have a genetic complement that is a mixture of the genetic complement of its two parents. (Fact 22.)

The Examiner's allegation that Appellant's clones are products of nature because a naturally occurring female was employed to make the clone (November 10, 2008, Office Action at 7-8, bridging ¶) is in error. Although nature may take a part in Appellant's invention, this does not convert Appellant's invention to a product of nature. If this were true, many, if not all, recombinant biological products would be considered "products of nature." For example, under the Examiner's reasoning, any recombinant drug that employed bacteria to "make" it would be a "product of nature." Certainly, this is not the law.

The relevant legal question under 35 U.S.C. § 101 is whether the hand of man is involved, and not whether nature is involved. *See Diamond v. Chakrabarty*, 447 U. S. 303 at 313. As recognized by the Supreme Court in *Diamond v. Chakrabarty*, a non-naturally occurring animal is patentable subject matter under 35 U.S.C. § 101. *Id.* Since claims 155-159 and 164 require a non-naturally occurring animal, these claims are directed to statutory subject matter.

In its prior decision, the Board asked the question: "Can it be that a copy of a preexisting thing is patentable?" (Decision at 13, first ¶.) Under 35 U.S.C. §

101, the question is whether a copy of a preexisting thing is *statutory subject matter*. The Federal Circuit has defined statutory subject matter under 35 U.S.C. § 101 very broadly:

> The use of the expansive term "any" in section 101 represents Congress's intent not to place any restrictions on the subject matter for which a patent may be obtained beyond those specifically recited in section 101 and the other parts of Title 35.... Thus, it is improper to read into section 101 limitations as to the subject matter that may be patented where the legislative history does not indicate that Congress clearly intended such limitations.

In re Alappat, 33 F.3d 1526, 1542 (Fed. Cir. 1994) (*en banc*). Thus, it is improper to create a new class of subject matter, a clone, that is excluded from protection under 35 U.S.C. § 101.

Does a clone fall into an existing category of subject matter excluded from patent protection by 35 U.S.C. § 101? The answer to this question is no. The categories of subject matter that are not eligible for patent protection (i.e., non-statutory subject matter) have been well-defined by the Supreme Court and the Federal Circuit. These categories are 1) claims directed to nothing more than abstract ideas (such as mathematical algorithms), 2) natural phenomena, and 3) laws of nature. *See, Diamond v. Diehr*, 450 U.S. 175, 185 (1981), *In re Alappat,* 33 F.3d at 1542. A clone does not fall into any of these categories. Since a clone is a living animal, it cannot be an abstract idea or a law of nature. Since nature

does not make clones, it cannot be a natural phenomenon. Since a clone does not fall into any of these categories, it must be statutory subject matter under 35 U.S.C. § 101.

An appropriate legal analysis does not support the Examiner's position that a clone is unpatentable over its donor animal under 35 U.S.C. § 101. Rather, legal precedent supports the opposite conclusion. Thus, although the Board's question ("Can it be that a copy of a preexisting thing is patentable?") is a valid question, it is properly addressed under the other sections of Title 35, particularly under 35 U.S.C. §§ 102 and 103, and not under 35 U.S.C. § 101.

There is no separate novelty requirement under 35 U.S.C. § 101

The Examiner concluded that the claimed cloned non-human mammals are not seen as "new" as required by 35 U.S.C. § 101. (November 10, 2008, Office Action at 7.) Similarly, in its previous decision in this case, the Board found that, "the term 'new' in § 101 cannot be ignored." (January 30, 2008, Decision ("Decision") at 13.) The Board asked: "[W]hat limitations of the claims distinguish the claimed product (a clone of a specified mammal) from other mammals of that type – in particular, from the donor of the nucleus?" (*Id.*)

The term "new" in 35 U.S.C. § 101 does not dictate an independent requirement of novelty or non-obviousness distinct from the more specific and detailed requirements of 35 U.S.C. §§ 102 and 103. As stated in a recent *en banc*

decision from the Federal Circuit:

> As the legislative history of § 101 indicates, Congress did not intend the "new and useful" language of § 101 to constitute an independent requirement of novelty or non-obviousness distinct from the more specific and detailed requirements of §§ 102 and 103, respectively. Diehr, 450 U.S. at 190-91. So here, it is irrelevant to the § 101 analysis whether Applicants' claimed process is novel or non-obvious.

In re Bilski, 545 F.3d 943, 958 (Fed. Cir. 2008). Thus, the Examiner's and Board's requirement for "newness" under 35 U.S.C. § 101 runs counter to decisions from the Federal Circuit and the Supreme Court stating that there is no independent requirement for newness in 35 U.S.C. § 101 distinct from §§ 102 and 103.

Appellant's clone is patentable over its donor mammal

Although not an appropriate issue under 35 U.S.C. §101, Appellant's clone is nonetheless patentable under 35 U.S.C. §§ 102 and 103 over its donor mammal First, Appellant's clone is not anticipated by its parental donor mammal. In its previous decision in this application, the Board admitted that the clone "will not be an exact copy of the 'parent.'" (Fact 1.) The Board understood that the term "clone" was not used in the claims on appeal to mean an exact copy. (Fact 18.) The Board conceded that environmental factors will result in physical differences between the clone and its parent. (Fact 2.) The Board further admitted that the clone and its parent will occupy a different space and time and will have phenotypic differences. (Fact 3.) Similarly, the Examiner admitted that the clone

and the donor animal will have differences. (Fact 4.) These differences are sufficient to negate a finding of anticipation of a clone by its parent under the proper legal analysis.

Anticipation under 35 U.S.C § 102 can be found only when the reference discloses ***exactly*** what is claimed; where there are differences between the reference disclosure and the claim, the rejection must be based on 35 U.S.C § 103, which takes differences into account. *Titanium Metals Corp. v. Banner*, 778 F.2d 775, 780 (Fed. Cir. 1985). Thus, anticipation is not shown by a prior art disclosure which is only "substantially the same" as the claimed invention. *Jamesbury Corp. v. Litton Industrial Products, Inc.*, 756 F.2d, 1556, 1560 (Fed. Cir. 1985). Rather, the ***identical*** invention must be shown in as complete detail as is contained in the claim. *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236 (Fed. Cir. 1989).

Following legal precedent, the fact that the clone and its parent are different, which the Board and the Examiner concede, precludes anticipation of Appellant's claims. As explained by the Court of Appeals for the Federal Circuit in overturning an anticipation rejection where there were differences between the claims and the prior art:

> Anticipation requires the presence in a single prior art disclosure of all elements of a claimed invention arranged as in the claim. *Soundscriber Corp. v. U.S.*, 360 F.2d 954, 960, 148 USPQ 298, 301 (Ct. Cl. 1966). A prior art disclosure that "almost" meets that standard

may render the claim invalid under §103; it does not "anticipate." *Connell et al. v. Sears, Roebuck & Co.*, 722 F.2d 1542, 1548 (1983).

Appellant's claims require that the clone is of a pre-existing, non-embryonic, donor mammal. Appellants explained in the specification that a clone is not identical to its parent. (Fact 7.) Thus, Appellant recognized that the claimed clone would have the same nucleus (i.e., chromosomal complement) as its parent, but that the clone and its parent would be different because of the fact that a different oocyte is used for each clone. Since a different oocyte is used for the clone and its parent, Appellant's clone and its parent cannot be identical.

The evidence provided to the Office during the prosecution of this application leaves no doubt that Appellant's clone is not identical to its parent. As explained in Prather et al., 1990, cloned offspring may vary phenotypically due to environment. (Fact 8.) Differences in mitochondrial DNA (from the oocyte) may also contribute to differences between a clone and its parent. (Fact 9.) Moreover, environmental factors, such a uterine environment, generate differences that prevent a clone and its parent from being phenotypically identical. (Fact 10.) Differences in phenotype may be manifested in many ways. For example, a clone and its parent may have phenotypic differences in color patterns caused by a different migration of melanocytes during development. (Fact 11.) Similarly, since the iris of every eye is unique, a clone will have a different pigmentation of

the iris as compared to its parent. (Facts 12 and 13.) Thus, a clone that contains the same set of chromosomes as a single parental mammal can be distinguished from the parental mammal due to these environmental influences. (Fact 14.) Also, the cloned mammal will have behavioral differences from the parental mammal. (Fact 15.)

Importantly, Appellant's clone will always be of a younger age than the donor mammal since the donor mammal must exist before the clone can exist. (Fact 16.) The ability of Appellant's clone to exist at a time later than its donor mammal, but have the same genetic complement, is a markedly different characteristic from any mammal found in nature. (Fact 33.) No mammal found in nature is a time-delayed genetic copy of either of its parent. (Fact 34.)

Appellant's clone provides an alternative, time-delayed source of nuclear genomic material of its donor mammal. (Fact 35.) This feature of Appellant's clone does not depend on the continued existence of the donor mammal. (Fact 36.) Appellant's clone can provide an alternative source of nuclear genomic material of its donor mammal, even if the donor mammal is dead. (Fact 37.) In this way, Appellant's clone allows the preservation of the genomic material of a particular mammal beyond the normal lifespan of that mammal. (Fact 38.)

Normally, when a mammal dies, its particular genomic composition is lost. (Fact 39.) Its progeny only contain one-half of each of its two parents' genomic

material. (Fact 40.) The genomic material of one parent is inextricably scrambled together with the other parent's genomic material to create the progeny. (Fact 41.) Appellant's clone avoids this permanent loss of a particular genomic composition. (Fact 42.) Appellant's clone provides the potential for the continuation of a particular genomic composition in a way that never occurs in nature. (Fact 43.) This cannot be considered a trivial difference from a clone's donor mammal, which does not have this potential. (Fact 44.) As a result, the clone and the parental mammal cannot be ***identical***. Accordingly, Appellant's clone is not anticipated by its parental donor mammal.

Furthermore, Appellant's clone is nonobvious over its parental donor mammal. Appellant's clone is time-delayed. (Fact 32.) Thus, Appellant's cloned mammal will always be of a younger age than the parental mammal. (Fact 16.) This difference in the age of the cloned mammal could not have been expected from the prior art because, prior to Appellant's invention, a clone of a pre-existing, non-embryonic, donor mammal never existed. No one would have ever had an expectation of success in producing such a clone. (Fact 47.) Rather, the production of a clone of a pre-existing, non-embryonic, donor mammal was not thought to be possible at that time. (Fact 48.) This unexpected result precludes a finding of obviousness of Appellant's clone. With non-living things, a copy is likely an obvious derivation. The same cannot be said of living mammals, which

exist for a fixed time period and whose offspring do not contain the same genetic complement due to sexual reproduction.

For all of the above reasons, the rejection of claims 155-159 and 164 under 35 U.S.C. § 101 should be reversed.

B. <u>Rejections under 35 U.S.C. § 102/103 over embryo cloned mammals</u>

The Examiner rejected claims 155-159 and 164 under 35 U.S.C. § 102(b) and/or 103(a) over several publications that teach embryo cloned cattle (Sims et al., 1993), sheep (McLaughlin et al., 1990), pigs (Prather et al., 1989), and goats (Yong et al., 1991). These references report embryo cloning, which is a process of cloning using nuclear transfer starting with an embryo as the nuclear donor. (Facts 53, 58, 63, and 68.) In this process, an embryo is destroyed to produce individual cells, which are then used for nuclear transfer to generate sibling clones. It is the Examiner's position that the mammals of the cited prior art anticipate or make obvious Appellant's claimed clones because there are no structural differences from the prior art mammals provided by Appellant's method of cloning.

Appellant previously pointed out where the Examiner erred in the Amendment filed March 30, 2008, on pages 9-13. Appellant's argument is that Appellant's claims require that the clone is of a pre-existing**, non-embryonic, donor** mammal. The cited prior art does not disclose a clone with this limitation. There is no **non-embryonic, donor** mammal in the cited prior art. Although the

cited prior art discloses clones, the clones of the prior art are clones of an *embryonic* donor, which is the opposite of "non-embryonic." Thus, the prior art does not disclose a clone of a **non-embryonic** donor mammal. As discussed *supra*, Appellant's claims are ***not*** product-by-process claims. Thus, the Examiner cannot ignore this limitation.

Anticipation requires the presence in a single prior art disclosure of all elements of a claimed invention arranged as in the claim. *Jamesbury Corp. v. Litton Industrial Products, Inc.*, 756 F.2d at 1560 (Fed. Cir. 1985). A reference which does not satisfy one limitation of a claim does not anticipate. *Id.*

Because Appellant's clone is a clone of a pre-existing, non-embryonic mammal, Appellant's claims requires two animals, namely, a pre-existing, **non-embryonic**, donor mammal and a clone of that donor mammal. (Fact 45.) This pair of animals has a special relationship in that one of the mammals is a clone of the other mammal. The cited references did not generate such a pair of mammals. In none of the cited references did a pre-existing, non-embryonic, parental mammal and a clone of that parental mammal exist. Thus, the prior art lacks this limitation of Appellant's claims. Since the prior art is missing this limitation of Appellant's claims, the cited prior art cannot anticipate them. *See Jamesbury,* 756 F.2d at 1560.

The references cited by the Office each describes clones made by

embryonic cloning procedures. That is, these references report a process of cloning using nuclear transfer starting with an embryo as the nuclear donor. (Facts 53, 58, 63, and 68.) The embryos used as the nuclear donors in the embryonic cloning procedures of the cited references were generated by normal sexual reproduction. (Facts 52, 57, 62, and 67.) Thus, these embryos were not genetically identical to either of its parents. (*See* Fact 25.) Furthermore, the embryos were destroyed during the embryonic cloning procedures. (Facts 54, 59, 64, and 69.) Consequently, the embryos used as the nuclear donors in the embryonic cloning procedures of the cited references were never "non-embryonic."

Furthermore, the non-embryonic parental mammals in the cited references would have been the two parents of each of the embryos used as the nuclear donors in the embryonic cloning procedures. The embryonic clones made in these references were not clones of either of these parental mammals, since sexual reproduction was used to generate the embryos used in the embryonic cloning procedures. Rather, these clones would have been a mixture of the genetic complement of their two parents, and thus the clones would not have had the same genetic complement as either of the parents. (Facts 19 and 22.) Consequently, the clones generated by the embryonic cloning procedures of the cited references were not a live-born clone of a pre-existing, ***non-embryonic***, donor mammal. These

prior art clones lack this element of Appellant's claims.

Moreover, the embryonic cloning procedures of the cited references preclude even the coexistence of the clone and the donor embryo. This is due to fact that, in the embryonic cloning procedures of the cited references, the embryonic donor was destroyed during the generation of the clone. (Facts 54, 59, 64, and 69.) In contrast, a live-born clone of a pre-existing, ***non-embryonic***, donor mammal as claimed can coexist with its non-embryonic donor. The generation of such a clone does not require destruction of the donor mammal in generating the clone.

Thus, Appellant's clones differ in many ways from the clones of the cited references. These differences preclude a finding of anticipation of Appellant's claims. *See Titanium*, 778 F.2d at 780.

The differences between Appellant's clones and the clones of the cited references also preclude a finding of obviousness of Appellant's claims. In determining the differences between the prior art and the claims, the question under 35 U.S.C. 103 is not whether the differences themselves would have been obvious, but whether the invention as a whole would have been obvious. *Stratoflex, Inc. v Aeriquip Corp.*, 713 F.2d 1530, 1537 (Fed Cir. 1983).

A live-born clone of a pre-existing, ***non-embryonic***, donor mammal as claimed is not taught or suggested by the cited references. Although the cited

references demonstrated that cloned mammals could be made from embryonic nuclear donor cells, it was unexpected that a clone of a non-embryonic nuclear donor mammal could be generated prior to Appellant's invention. (Fact 47.) Prior to Appellant's invention, it was not considered to be possible to produce a clone of a non-embryonic mammal. (Fact 48.) Thus, Appellant's clone could not have been obvious at the time the application was filed.

Since there must be a reasonable expectation of success to support a conclusion of obviousness, what was thought to be impossible cannot be obvious. *See, e.g., In re Spormann*, 363 F.2d 444, 448 (CCPA 1966). Accordingly, the rejection should be reversed.

C. Rejections under 35 U.S.C. § 102/103 over IVF mammals

The Examiner rejected claims 155-159 and 164 under 35 U.S.C. § 102 or § 103 as being anticipated by or obvious over a number of references teaching cattle (Zinn), sheep (Aldrich), pigs (Matte), and goats (Ortega-Reyes) produced by in vitro fertilization. Appellant has not previously responded to this rejection.

Appellant's argument is that the cited references do not disclose exactly what is claimed. Rather, the cited references are missing an element recited in Appellant's claims. The cited prior art references do not disclose a **clone**. This limitation of Appellant's claim precludes Appellant's clone from being anticipated by the cited references. *See Jamesbury,* 756 F.2d at 1560.

The references cited by the Office each describes mammals made by in vitro fertilization ("IVF") procedures. Thus, the mammals produced in these references were generated by using an egg and a sperm, albeit using in vitro procedures. The mammals made in these references were not **clones** of either of these parental mammals, since sexual reproduction was used to generate the embryos used in the IVF procedures. Rather, these mammals generated by IVF would have been a mixture of the genetic complement of their two parents, and thus the mammals would not have had the same genetic complement as either of the parents. (Fact 22.) Thus, these mammals were not identical to either of its parents, but only 50% identical to each of them. (*See* Fact 25.) Consequently, the mammals generated by the in vitro fertilization procedures of the cited references were not a live-born **clone** of a pre-existing, non-embryonic, donor mammal. The cited prior art references lack this element of Appellant's claims.

A live-born clone of a pre-existing, non-embryonic, donor mammal as claimed is a time-delayed, inexact copy of a non-embryonic mammal. (Fact 32.) The claimed clone requires two animals, namely, a pre-existing, non-embryonic, donor mammal and a clone of that donor mammal. (Fact 45.) The cited references did not generate such a pair of mammals. Thus, the prior art lacks this limitation of Appellant's claims. Since the prior art is missing this limitation of Appellant's claims, the cited prior art cannot anticipate them. *See Jamesbury,* 756 F.2d at

1560.

The differences between Appellant's clones and the mammals of the cited references also preclude a finding of obviousness of Appellant's claims. A live-born clone of a pre-existing, non-embryonic, donor mammal as claimed is not taught or suggested by the cited references. Prior to Appellant's invention, the generation of a live-born clone of a pre-existing, non-embryonic, donor mammal would have been expected to be impossible. (Facts 47 and 48.) Since there must be a reasonable expectation of success to support a conclusion of obviousness, what was thought to be impossible cannot be obvious. *See, e.g., In re Spormann*, 363 F.2d at 448.

Appellant points out that the previous Board Decision in this application concluded that Appellant's claims to horses and rats were not anticipated or obvious over other previously cited references, stating: "The Examiner has not relied on any evidence that a horse or rat having the same nuclear genetic code as a previously existing horse or rat existed or was enabled prior to Campbell's disclosure." (Facts 24-27.) The same is true here. None of the cited references shows that a mammal having the same nuclear genetic code as a previously existing mammal existed or was enabled prior to Campbell's disclosure. Accordingly, the rejection is in error and should be reversed.

The Examiner further questions: "How do you distinguish from mammal

produced by mating from a clone, or a mammal produced by IVF from a clone?" (November 10, 2008, Office Action at 17, first full ¶.) First, this is a question relevant to infringement, not one of anticipation or obviousness. Second, Appellant's claims are not product-by-process claims. Thus, they do not require that the clone is "produced by nuclear transfer," but only that it is a clone. Third, one skilled in the art could readily determine which animal was a clone by comparing the DNA of the progeny to the parent animals. Only the clone would have the same genetic complement as its parent, i.e., the nuclear donor. The IVF produced mammal would have a mixture of the genetic complement of its egg donor and sperm donor. This could readily be determined by DNA analysis using routine techniques in the art.

Appellant's clones have a feature that sets them apart from all of the mammals in the prior art. Appellant's clones are time-delayed copies of pre-existing animals. (Fact 32.) Since Appellant's clone is an inexact copy of the pre-existing parental donor mammal, the clone has the same genetic complement as the parental donor mammal. (Fact 21.) Only Appellant's clone will contain the same set of chromosomes as a single non-embryonic parental mammal. (Fact 19.)

Appellant's clone is unlike any mammal produced by a process involving sexual reproduction. (Fact 20.) This would include, mammals made by mating, by IVF, or produced by nuclear transfer from an embryonic cell. The set of

chromosomes of a clone of a pre-existing, non-embryonic, donor mammal is obtained from a single parental donor mammal. (Fact 21.) The set of chromosomes from any mammal produced by a process involving sexual reproduction, including a mammal produced by nuclear transfer from an embryonic cell, comes from two parental mammals, one male and one female. (Fact 22.) This difference allows Appellant's clone to preserve the genetic information of the parental donor mammal without dilution. (Fact 23.)

The differences between Appellant's clones and the mammals of the cited references preclude a finding of anticipation or obviousness of Appellant's claims. Accordingly, the rejection should be reversed.

Respectfully submitted,

Law Office of Salvatore Arrigo

Dated: September 8, 2009

By: /Salvatore J. Arrigo/

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APPENDIX

Claims Section

Claims 1-154 (Canceled).

155. (Rejected) A live-born clone of a pre-existing, non-embryonic, donor mammal, wherein the mammal is selected from cattle, sheep, pigs, and goats.

156. (Rejected) The clone of claim 155, wherein the mammal is a cattle.

157. (Rejected) The clone of claim 155, wherein mammal is a sheep.

158. (Rejected) The clone of claim 155, wherein the mammal is a pig.

159. (Rejected) The clone of claim 155, wherein the mammal is a goat.

160-163. (Canceled).

164. (Rejected) The clone of any of claims 155-159, wherein the donor mammal is non-foetal.

Claim Support and Drawing Analysis Section

155. A live-born clone of a pre-existing, non-embryonic, donor mammal **{page 3, lines 12-16; page 5, lines 14-19}**, wherein the mammal is selected from cattle**{page 3, lines 12-16; page 5, lines 14-19}**, sheep **{page 3, lines 12-16; page 5, lines 14-19}**, pigs **{page 3, lines 12-16; page 5, lines 14-19}**, and goats **{page 3, lines 12-16; page 5, lines 14-19}**.

There is no limitation illustrated in a drawing.

Means or step plus function analysis section

There are no means or step plus function limitations in the claims to be considered.

#25 Dec 4/25/03

PATENT
(700/202D)
Attorney Docket No. 112800.401

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Keith Henry Stockman Campbell et al.	Group Art Unit: 1632
Serial No.: 09/225,233	Examiner: D. Crouch
Filed: January 4, 1999	

For: QUIESCENT CELL POPULATIONS FOR NUCLEAR TRANSFER

Commissioner for Patents
Washington, DC 20231

Sir:

DECLARATION OF DR. DAVID WELLS UNDER 37 C.F.R. § 1.132

I, David Wells, declare that:

1. Since 1992, I have held the position of Research Scientist, AgResearch, Ruakura, New Zealand. I am a principal researcher in the field of nuclear transfer with embryonic and somatic cells and have been involved in the establishment of embryonic stem (ES) cell technology at AgResearch. In part, my research involves cloning livestock animals, particularly sheep and cattle, from cultured cells using nuclear transfer into oocytes. I have conducted many studies using nuclear transfer into oocytes and developing the resultant embryos into fetuses and animals. A copy of my Curriculum Vitae is attached hereto.

2. From 1987 to 1988, I held the position of Research Scientist, MAF Technology, Ruakura, New Zealand. During my employment with MAF Technology I developed an embryo splitting technique, which was integrated into a large scale

multiple ovulation and embryo transfer program conducted at Hopu Hopu Quarantine Research Station in New Zealand. My research involved cloning sheep by embryo splitting. This involved embryo manipulation and culture and the development of identical twin animals by transfer into hosts.

3. In 1991, I graduated with a Doctor of Philosophy from the University of Edinburgh, UK. I conducted the research for my PhD thesis at the Department of Reproduction and Development, AFRC Institute of Animal Physiology and Genetics Research, Roslin Institute, and the Department of Genetics, at the University of Edinburgh. During my PhD program, I worked in Dr. Ian Wilmut's laboratory and developed competence in embryonic stem (ES) cell isolation. My work involved using isolated ES cells to generate germline chimeras by embryo manipulation and culture and the development of animals.

4. I have published numerous manuscripts in the area of cloning livestock animals from cultured cells using nuclear transfer. Two representative manuscripts are: "Production of cloned lambs from an established embryonic cell line: a comparison between in vivo- and in vitro-matured cytoplasts" published in *Biology of Reproduction* 57: 385-393 (1997) and "Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells" published in *Biology of Reproduction* 60: 996-1005 (1999).

5. I have read an article by McLaughlin et al. in *Reproduction Fertil. Develop.* 2, 619-622 (1990), a copy of which is attached hereto.

6. McLaughlin et al. discusses the production of embryos and lambs by nuclear transfer using sheep embryonic cells as nuclear donors.

A1

7. Based on McLaughlin et al. and on my experience in cloning mammals, the embryos and sheep of McLaughlin et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and sheep of McLaughlin et al. were generated from sheep embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and sheep of McLaughlin et al. had two parents, one male and one female. The embryos and sheep of McLaughlin et al. had a chromosomal donation from each of these parents. Therefore, embryos and sheep of McLaughlin et al. do not contain the same set of chromosomes as either of their parents.

8. Based on McLaughlin et al. and on my experience in cloning mammals, the embryos and sheep of McLaughlin et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

9. I have read an article by Prather et al. in *Biology of Reproduction* 41, 414-418 (1989), a copy of which is attached hereto.

10. Prather et al. discusses the production of embryos and pigs by nuclear transfer using pig embryonic cells as nuclear donors.

11. Based on Prather et al. and on my experience in cloning mammals, the embryos and pigs of Prather et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and pigs of Prather et al. were generated from pig embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and pigs of Prather et al. had two parents, one male and one female. The embryos and pigs of

Prather et al. had a chromosomal donation from each of these parents. Therefore, embryos and pigs of Prather et al. do not contain the same set of chromosomes as either of their parents.

12. Based on Prather et al. and on my experience in cloning mammals, the embryos and pigs of Prather et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

13. I have read an article by Yong et al. in *Theriogenology* 35, 299 (1991), a copy of which is attached hereto.

14. Yong et al. discusses the production of embryos and goats by nuclear transfer using goat embryonic cells as nuclear donors.

15. Based on Yong et al. and on my experience in cloning mammals, the embryos and goats of Yong et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and goats of Yong et al. were generated from goat embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and goats of Yong et al. had two parents, one male and one female. The embryos and goats of Yong et al. had a chromosomal donation from each of these parents. Therefore, embryos and goats of Yong et al. do not contain the same set of chromosomes as either of their parents.

16. Based on Yong et al. and on my experience in cloning mammals, the embryos and goats of Yong et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various

techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

17. I have read an article by Cheong et al. in *Biology Reproduction* 48, 958-963 (1993), a copy of which is attached hereto.

18. Cheong et al. discusses the production of embryos and mice by nuclear transfer using mouse embryonic cells as nuclear donors.

19. Based on Cheong et al. and on my experience in cloning mammals, the embryos and mice of Cheong et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and mice of Cheong et al. were generated from mouse embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and mice of Cheong et al. had two parents, one male and one female. The embryos and mice of Cheong et al. had a chromosomal donation from each of these parents. Therefore, embryos and mice of Cheong et al. do not contain the same set of chromosomes as either of their parents.

20. Based on Cheong et al. and on my experience in cloning mammals, the embryos and mice of Cheong et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

21. I have read an article by Yang et al. in *Biology Reproduction* 47, 636-643 (1992), a copy of which is attached hereto.

22. Yang et al. discusses the production of embryos and rabbits by nuclear transfer using rabbit embryonic cells as nuclear donors.

23. Based on Yang et al. and on my experience in cloning mammals, the embryos and rabbits of Yang et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and rabbits of Yang et al. were generated from rabbit embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and rabbits of Yang et al. had two parents, one male and one female. The embryos and rabbits of Yang et al. had a chromosomal donation from each of these parents. Therefore, embryos and rabbits of Yang et al. do not contain the same set of chromosomes as either of their parents.

24. Based on Yang et al. and on my experience in cloning mammals, the embryos and rabbits of Yang et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

25. I have read an article by Sims et al. in *Proc. Natl. Acad. Sci.* 90, 6143-6147 (1993), a copy of which is attached hereto.

26. Sims et al. discusses the production of embryos and bovines by nuclear transfer using bovine embryonic cells as nuclear donors.

27. Based on Sims et al. and on my experience in cloning mammals, the embryos and bovines of Sims et al. do not contain the same set of chromosomes as a single parental mammal. This is because the embryos and bovines of Sims et al. were generated from bovine embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the embryos and bovines of Sims et al. had two parents, one male and one female. The embryos

and bovines of Sims et al. had a chromosomal donation from each of these parents. Therefore, embryos and bovines of Sims et al. do not contain the same set of chromosomes as either of their parents.

28. Based on Sims et al. and on my experience in cloning mammals, the embryos and bovines of Sims et al. could be distinguished from an embryo or mammal that contains the same set of chromosomes as a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

29. I have read WO 95/17500 published June 29, 1995, of Stice et al., a copy of which is attached hereto.

30. Stice et al. discusses the production of genetically modified embryos and mammals by nuclear transfer using genetically modified embryonic cells as nuclear donors.

31. Based on Stice et al. and on my experience in cloning mammals, the genetically modified embryos and mammals of Stice et al. did not receive their chromosomes from a single parental mammal. This is because the genetically modified embryos and mammals of Stice et al. were generated from genetically modified embryonic cells. The embryos from which these cells were derived were the product of normal sexual reproduction. Consequently, the genetically modified embryos and mammals of Stice et al. had two parents, one male and one female. The genetically modified embryos and mammals of Stice et al. had a chromosomal donation from each of these parents. Therefore, the genetically modified embryos and mammals of Stice et al. did not receive their chromosomes exclusively from one of their parents.

32. Based on Stice et al. and on my experience in cloning mammals, the genetically modified embryos and mammals of Stice et al. could be distinguished from an embryo or mammal that receives its chromosomes from a single parental mammal by various techniques. Differences and identities in chromosomes could be readily determined, for example, using the well-known technique of genetic analysis.

33. Based on my experience with mammals cloned by nuclear transfer and mammals propagated by sexual reproduction, the source of a mammal's chromosomes can be readily determined using genetic analysis. By using genetic analysis, whether a mammal is cloned asexually by somatic cell nuclear transfer or propagated by sexual reproduction, including a mammal produced by nuclear transfer from an embryonic cell, can be determined by comparing the chromosomal DNA of the mammal to that of its parent(s). Only a mammal cloned by somatic cell nuclear transfer will contain the same set of chromosomes as a single parental mammal.

34. Based on my experience with mammals cloned by nuclear transfer and mammals propagated by sexual reproduction, a mammal cloned by somatic cell nuclear transfer is unlike any mammal produced by a process involving sexual reproduction, including a mammal produced by nuclear transfer from an embryonic cell. The set of chromosomes of a mammal cloned by somatic cell nuclear transfer is obtained from a single parental mammal. The set of chromosomes from any mammal produced by a process involving sexual reproduction, including a mammal produced by nuclear transfer from an embryonic cell, comes from two parental mammals, one male and one female. This feature allows the cloned mammal to preserve the genetic information of the parental mammal without dilution.

35. Based on my experience with cloned mammals, a mammal that contains the same set of chromosomes as a single parental mammal can be distinguished from the parental mammal due to environmental influences. First, the cloned mammal will always be of a younger age than the parental mammal. Second, the cloned mammal will have a variety of phenotypic differences from the parental mammal, for example, differences in fur and skin pigmentation. Third, the cloned mammal will have behavioral differences from the parental mammal.

36. The undersigned further declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing therefrom.

Dated: 5th February 2003

By: D. W. Wells
David Wells, Ph.D

PATENT
Customer No. 76,392
Attorney Docket No. SP-01-US-DIV-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Campbell et al.	)	Group Art Unit: 1632
	)	
Application No.: 09/225,233	)	Examiner: D. Crouch
	)	
Filed: January 4, 1999	)	Confirmation No.: 2711
	)	
For: QUIESCENT CELL POPULATIONS FOR NUCLEAR TRANSFER	)	
	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF IRINA A. POLEJAEVA, Ph.D, UNDER 37 C.F.R. § 1.132

I, Irina A. Polejaeva, declare that:

1. Since December of 2003, I have held the position of Vice President of Assisted Reproductive Technology & Chief Scientific Officer, ViaGen Inc. I held the position of Director of Assisted Reproductive Technology between October 2002 and November 2003. At ViaGen Inc, I have been involved in cloning horses, cattle, and pigs using somatic cells. A copy of my CV is attached.

2. I understand that ViaGen Inc. is a licensee of the above-referenced patent application from Start Licensing Inc.

3. I further understand that the prosecution of the above-referenced patent application is controlled by Start Licensing Inc., a joint venture between Geron Corporation and Exeter Life Sciences Inc.

4. I further understand that ViaGen Inc. is a subsidiary of Exeter Life Sciences Inc.

5. In 1998-2002, I was the Head of Cell Biology, Senior Scientist, and Porcine Nuclear Transfer Project Manager at PPL Therapeutics Inc. I was responsible for planning, budgeting, training, coordination, evaluating, and reporting of all aspects of research projects in the area of Cell Biology and Porcine Embryology. This included developing nuclear transfer procedures in order to produce transgenic mammals and improving efficiency of nuclear transfer procedures by optimizing oocyte activation. I was responsible for designing appropriate tissue culture conditions for generation and propagation of primary bovine and porcine cells for nuclear transfer programs.

6. In 1996-1998, I was a Scientist and Project Manager for porcine stem cell project at PPL Therapeutics Inc. I was responsible for planning, conducting, supervising, and summarizing data on a porcine stem cell project.

7. In 1993-1996, I was a postdoctoral fellow at Utah State University. During my research at Utah State University, I established bovine, mink, and ovine ES cell lines. I optimized culture conditions for the isolation and maintenance of bovine ES cells. I was further involved in a project that measured DNA methylase activity during various passages of bovine ES cells.

8. In 1993, I was a Scientist in the Department of Biotechnology, National Research Institute of Animal Science, Russia. I was involved in a project aimed at establishing bovine ES cells. I established primary cell cultures from lung, liver, kidney and connective tissue of transgenic rabbits for investigating bovine growth hormone gene expression.

9. In 1989-1992, I was a student at the Laboratory of Cell Engineering (lab of Dr. N. Strelchenko) at the National Institute of Animal Science, Russia. I was involved in several projects aimed at establishing mouse ES cells and the production of transgenic murine ES cells using a retroviral vector. I established primary cultures of mammalian and insect cells, prepared feeder layers for ES cell maintenance, maintained and transformed embryonic stem cells, and mastered procedures for freezing and thawing cells as well as karyotyping cell lines. In June 1993, I was granted a Ph.D. in Developmental Biology/Embryology, from the Department of Biotechnology, National Research Institute of Animal Science, Dubrovitsy, Moscow region, Russia.

10. In June 1985, I obtained a Master of Science in Animal Science at the Kubanski Agricultural Institute, Krasnodar, Russia.

11. I have published numerous manuscripts in the area of cloning mammals from somatic cells using nuclear transfer. Representative manuscripts are: Walker S.C., Christenson R.K., Ruiz R.P., Reeves D.E., Pratt S.L., Arenivas F., Williams N.E., Bruner B.L., Polejaeva I.A., Comparison of meat composition from offspring of cloned and conventionally produced boars, *Theriogenology* 67:178-184 (2007); Irina A. Polejaeva, Pig cloning: advances and applications, *Reproduction, Supplement* 58: 293-300 (2001); and Irina A. Polejaeva, Shu-Hung Chen, Todd D. Vaught, Raymond L. Page, June Mullins, Yifan Dai, Jeremy Boone, Shawn Walker, Dave Ayares, Alan Colman and Keith H. S. Campbell, Cloned Piglets Produced by Nuclear Transfer from Adult Somatic Cells, *Nature* 407: 86-90 (2000).

12. Through my experience, I have gained knowledge of many aspects of mammalian biology, including nuclear transfer (cloning) technology; oocyte activation

and early embryo development after nuclear transfer; mechanism of nuclear reprogramming; early events of mammalian development; gene transfer based on the use of somatic cell nuclear transfer and embryonic stem cell technologies; gene targeting in ES cells and somatic cells; *in vitro* differentiation of cells; and the developmental biology and embryology of mammals.

Campbell's U.S. Patent Application No. 09/225,233

13. I have read Campbell's U.S. Patent Application No. 09/225,233 ("the Campbell '233 application"), a copy of which is attached hereto.

14. The Campbell '233 application states: "It has now been found that quiescent cells, that is to say cells that are not actively proliferating by means of the cell cycle, can advantageously used as nuclear donors in the reconstitution of an animal embryo. Such animals may then be allowed to develop to term." (At 4, lines 27-31.)

15. Based on my reading of the Campbell '233 application, the basic technique of somatic cell nuclear transfer taught by the Campbell '233 application involves transferring the nuclear genetic material of a quiescent somatic cell (the nuclear donor cell) into a suitable recipient cell.

16. In the Campbell '233 application, a nuclear donor cell can be in the G_0 stage of the cell cycle. (At 9, lines 14-28.)

17. In the Campbell '233 application, a suitable recipient cell can be an enucleated oocyte in the metaphase II stage of the cell cycle. (At 11, lines 22-35.)

18. The Campbell '233 application teaches many permutations of this technique. For example, on pages 9-13, the Campbell '233 application teaches various

techniques for introducing the nuclear genetic material of a somatic cell into a recipient cell.

19. Similarly, on pages 14-15, the Campbell '233 application teaches various techniques for activating a reconstituted embryo.

20. Also, on pages 15-16, the Campbell '233 application teaches various techniques for increasing the number of cloned mammals, including the technique of serial nuclear transfer. Serial nuclear transfer involves performing an additional nuclear transfer using cells generated from the first nuclear transfer as donors for a second nuclear transfer.

21. The Campbell '233 application points out that most of the reconstituted embryos do not reprogram. (At 16, lines 5-9.)

22. This means that somatic cell nuclear transfer is an inefficient process. Many reconstituted embryos need to be created to generate a single viable embryo.

23. Based on the Campbell '233 application, I would have known in February of 1997 that somatic cell cloning of mammals, including pigs, by nuclear transfer was an inefficient process.

Polejaeva et al. (2000)

24. I have read an article by I.A. Polejaeva, S. Chen, T.D. Vaught, R.L. Page, J. Mullins, S. Ball. Y. Dal, J. Boone, S. Walker, D. Ayares, A. Colman, and K.H.S. Campbell entitled "Cloned pigs produced by transfer from adult somatic cells" in *Nature* 407:86-90 (2000)("Polejaeva et al. (2000)"), a copy of which is attached hereto.

25. I am one of the authors of Polejaeva et al. (2000).

26. Polejaeva et al. (2000) reports using a serial nuclear transfer technique to generate cloned pigs, in which the nucleus from the reconstructed embryo was transferred to an enucleated zygote for further development.

27. I was involved in the experiments that generated cloned pigs as reported in Polejaeva et al. (2000).

28. In my opinion, the serial nuclear transfer technique that we used to clone pigs in Polejaeva et al. (2000) is not required to generate cloned pigs. My opinion is based on my personal experience cloning pigs, and on many reports of successful cloning of pigs without this serial nuclear transfer technique.

29. In Polejaeva et al. (2000), we transferred 72 nuclear transfer embryos to a surrogate, which resulted in five live-born pigs. (At 87, Table 1.)

30. In Polejaeva et al. (2000), we indicated that greater than 4 good quality embryos are required to induce and maintain pregnancy in pigs, citing a paper published in 1966. (At 86, col. 2, paragraph 3.)

31. I have read the 1966 paper referenced in Polejaeva et al. (2000). It is an article by Polge et al. entitled "The effect of reducing the number of embryos during early stages of gestation on the maintenance of pregnancy in the pig" in *J. Reprod. Fert.* 12, 395-396 (1966)("Polge et al. (1966)"), a copy of which is attached hereto.

32. Polge et al. (1966) states: "These results suggest that more than four embryos are required in the pig in order to establish and maintain pregnancy." (At 397, first full paragraph.)

33. Thus, it was known prior to February of 1997 that more than four viable embryos in a single surrogate pig would be required after nuclear transfer to maintain pregnancy in the pig and result in live-born clones.

34. It would have been known from the Campbell '233 application that only a small fraction of nuclear transfer embryos were viable. (At 16, lines 5-9.)

35. Based on the Campbell '233 application and Polge et al. (1966), it would have been expected in February of 1997 that many nuclear transfer embryos would need to be transferred to a single pig to achieve success.

36. In Polejaeva et al. (2000), we indicated that, with the cell lines that did not result in live-born pigs, between 22 and 100 reconstructed embryos were transferred to each recipient female. (At 87, Table 1.)

37. In my opinion, the lack of live-born pigs with these cell lines may be due to not transferring enough reconstructed embryos to assure success. My opinion is based on my experience in cloning mammals, the required efficiency to produce live-born pigs, and the number of embryos transferred in Polejaeva et al. That is, we only transferred between 22 and 100 reconstructed embryos to each recipient female. I note that we transferred the reconstructed embryos approximately 1 day after nuclear transfer, at a time point when a smaller percentage of the embryos are likely to survive to term than those transferred at later time points. Since only a small percentage of reconstructed embryos actually develop into a live-born mammal and this efficiency can be less than one live-born mammal for every 300 reconstructed embryos (i.e., less than 0.33%;), the lack of any live births with these cell lines does not surprise me.

38. The '233 application provides a good example of the low efficiency of the production of viable embryos by nuclear transfer, teaching 1 live birth for 277 reconstructed sheep embryos. (At 34, table 4.)

39. Moreover, based on Table 1 of Polejaeva et al. (2000), it is evident that multiple hosts may be required in addition to transferring sufficient embryos. That is, one would not expect every recipient to get pregnant because of differences in the specific hosts.

40. We decided to pursue serial nuclear transfer to clone pigs because we thought that this approach would increase efficiency.

41. In fact, I have cloned pigs without serial nuclear transfer.

42. In my experience, transfer of 100-150 nuclear transfer pig embryos is normally sufficient to maintain pregnancies in pigs and produce cloned pigs by somatic cell nuclear transfer.

43. In my experience, transfer of less than 100-150 nuclear transfer pig embryos may be insufficient to maintain pregnancies in pigs and produce cloned pigs by somatic cell nuclear transfer.

44. In my experience, if the viability of the embryos is increased, for example, by the serial nuclear transfer technique described in Polejaeva et al. (2000), less than 100-150 nuclear transfer pig embryos may be sufficient to maintain pregnancies in pigs and produce cloned pigs by somatic cell nuclear transfer.

45. Many groups have published reports of cloning pigs without using serial nuclear transfer, including Onishi et al. (2000), Betthauser et al. (2000), and Lai et al. (2002).

Successful Cloning of Pigs without Serial Nuclear Transfer

46. I have read an article by A. Onishi, M. Iwamoto, T. Akita, S. Mikawa, K. Takeda, T. Awata, H. Hanada, and A.C.F. Perry entitled "Pig Cloning by Microinjection of Fetal Fibroblast Nuclei" in *Science* 289:188-189 (2000)("Onishi et al. (2000)"), a copy of which is attached hereto.

47. Onishi et al. (2000) successfully cloned pigs by nuclear transfer using somatic cells as nuclear donors. Onishi et al. (2000) did not use the serial nuclear transfer technique we used in Polejaeva et al (2000).

48. Onishi et al. (2000) states: "pigs typically require at least four fetuses for a successful pregnancy." (At 1188, col. 3, last full paragraph.)

49. Onishi et al. (2000) transferred 36 nuclear transfer embryos to the oviduct of a female surrogate to achieve a single offspring. (At 1189, col. 1-2, bridging paragraph.)

50. The cloning procedure used by Onishi et al. (2000) to successfully clone pigs using somatic cells follows the basic procedure in the Campbell '233 application.

51. The Campbell '233 application teaches that recipient cells for use in cloning can be enucleated oocytes in the metaphase II stage of the cell cycle. (At 12, lines 4-7.)

52. Onishi et al. (2000) used enucleated oocytes in the metaphase II stage of the cell cycle. (At 1190, col. 1, "18.")

53. The Campbell '233 application teaches that the donor nucleus can be in the G_0 stage at the time of nuclear transfer. (At 12, lines 15-17.)

54. Onishi et al. (2000) used confluent fibroblast cells in the G_0 stage of the cells cycle as nuclear donors. (At 1190, col. 1, "14.")

55. The Campbell '233 application teaches that the donor nuclei can by introduced into the enucleated oocyte by microinjection. (At 13, lines 31-35.)

56. Onishi et al. (2000) used microinjection to transfer fibroblast nuclei into enucleated oocytes. (At 1190, col. 2-3, "19.")

57. The Campbell '233 application teaches activating the reconstructed embryos subsequent to nuclear transfer. (At 11, lines 28-30.)

58. Onishi et al. (2000) maintained the oocytes for 4 hours after transfer before activating them. (*Id.*)

59. I have read an article by J. Betthauser, E. Forsberg, M. Augenstein, L. Childs, K. Eilertsen, J. Enos, T. Forsythe, P. Golueke, G. Jurgella, R. Koppang, T. Lesmeister, K. Mallon, G. Mell, P. Misica, M. Pace, M. Pfister-Genskow MN. Strelchenko, G. Voelker, S. Watt, S. Thompson, and M. Bishop entitled "Production of cloned pigs from in vitro systems" in *Nature Biotechnology* 18:1055-1059 (2000)("Betthauser et al. (2000)"), a copy of which is attached hereto.

60. Betthauser et al. (2000) successfully cloned pigs by nuclear transfer using somatic cells as nuclear donors. Betthauser et al. (2000) did not use the serial nuclear transfer technique we used in Polejaeva et al (2000).

61. Betthauser et al. (2000) transferred 115-164 nuclear transfer embryos to each recipient to achieve cloned pigs. (At 1057, col. 1, second full paragraph.)

62. Betthauser et al. (2000) concludes: "The large number of embryos (115-164; Table 1) that were used to produce the cloned piglets and additional pregnancies described here is consistent with low viability of NT embryos." (*Id.*)

63. I have read an article by L. Lai, D. Kolber-Simonds, K.W., Park, H.T. Cheong, J.L. Greenstein, G.S. Im, M. Samuel, A. Bonk, A. Rieke, B.N. Day, C.N. Murphy, D.B. Carter, R.J., Hawley and R.S. Prather entitled "Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning" in *Science* 295:1089-92 (2002)("Lai et al. (2002)"), a copy of which is attached hereto.

64. Lai et al. (2002) successfully cloned pigs by nuclear transfer using somatic cells as nuclear donors. Lai et al. (2002) did not use the serial nuclear transfer technique we used in Polejaeva et al (2000).

65. Lai et al. (2002) states: "A minimum of four viable embryos is required for establishment of pregnancy in pigs (27)." (At 1090, col. 3, first full paragraph.)

66. Lai et al. (2002) performed 20 transfers of only NT-derived embryos to unmated surrogates, which resulted in two pregnancies continuing to term. (At 1091, col. 1, first full paragraph.) In the two cases that went to term, 92 and 130 nuclear transfer embryos were transferred. (At 1090. Table 1.)

67. Based on Onishi et al. (2000), Betthauser et al. (2000), Lai et al. (2002), and my own experience cloning pigs, it is my opinion that serial nuclear transfer technique we used in Polejaeva et al. (2000) is not required for somatic cell cloning of pigs by nuclear transfer.

68. Based on Polejaeva et al. (2000), Onishi et al. (2000), Betthauser et al. (2000), Lai et al. (2002), and my own experiences cloning pigs, it is my opinion that

somatic cell cloning of pigs by nuclear transfer in February of 1997 would not have required any technique that is not taught by the Campbell '233 application, or by the prior art.

69. Based on Polge et al. (1966), I would have further understood that somatic cell cloning of pigs by nuclear transfer would require having at least 4 viable pig embryos to maintain pregnancy.

70. Thus, in February of 1997, I would have expected that many nuclear transfer embryos would need to be transferred to a single pig to achieve successful somatic cell cloning of pigs.

71. Nonetheless, in February of 1997, I would have expected that transfer of sufficient nuclear transfer embryos to a single pig would result in successful somatic cell cloning of pigs.

72. The results of Polejaeva et al. (2000), Onishi et al. (2000), Betthauser et al. (2000), Lai et al. (2002), and my own experiences cloning pigs confirm these expectations.

73. In my opinion, the somatic cell cloning of pigs exemplifies the broad applicability of the cloning techniques set forth in the Campbell '233 application. Many groups readily achieved the goal of cloning pigs.

74. Based on Polejaeva et al. (2000), Onishi et al. (2000), Betthauser et al. (2000), Lai et al. (2002), and my own experiences cloning pigs, one can make many different modifications to the cloning techniques set forth in the Campbell '233 application, and still successfully clone pigs by somatic cell nuclear transfer.

75. Based on Polejaeva et al. (2000), Onishi et al. (2000), Betthauser et al. (2000), Lai et al. (2002), and my own experiences cloning pigs, the important aspects of the successful cloning of pigs are set forth in the Campbell '233 application and the prior art.

76. Based on Polejaeva et al. (2000), Onishi et al. (2000), Betthauser et al. (2000), Lai et al. (2002), and my own experiences cloning pigs, I would have expected that successful cloning of pigs could have been achieved with only routine experimentation in February of 1997 using the disclosure in the Campbell '233 application together with the knowledge in the art at that time.

77. It would have been routine in February of 1997 to design experiments based on the disclosure in the Campbell '233 application and the knowledge in the art at that time and to repeat the nuclear transfer experiments many times with hundreds of embryos and transfer those embryos to many different hosts. Nonetheless, many laboratories might not have the financial resources or manpower for such experiments.

Westhusin and Pennisi

78. I have read an article by M.E. Westhusin, C.R. Long, T. Shin, J.R. Hill, C.R. Looney, J.H. Pryor, and J.A. Piedrahita entitled "Cloning to reproduce desired genotypes" in *Theriogenology* 55:35-49 (2001)("Westhusin et al. (2001)"), a copy of which is attached.

79. Westhusin et al. (2001) makes a number of comments on the work effort required and the probability for producing a clone. (At 35-36.) Westhusin points out that the efficiency of each step of cloning varies among species, and that this affects the ease of which a particular animal can be cloned. (At 36.) Westhusin further points out

that the production of cloned pigs is very inefficient, and that the differences in the ability to clone a specific animal are simply a result of the time and resources that have thus far been invested in research for these species. (At 39.) Westhusin concludes that there is no solid evidence that suggests cloning will be limited to only a few specific animals, and in fact, most data collected to date suggests cloning will be applicable to a wide variety of different animals. (At 35.)

80. In my opinion, based on my experience in cloning mammals, there was no solid evidence in 2001 that any mammalian species could not be cloned, and there is no solid evidence today that any mammalian species cannot be cloned.

81. Based on the above passages, I understand that cloning is an inefficient process and a large number of oocytes may need to be reconstructed to achieve success. The probability for producing a clone increases proportionally with the number of oocytes reconstructed, but so does the "work effort," as well as the cost. Based on my experience in cloning mammals, the challenge for most laboratories in cloning mammals is one of having sufficient manpower and financial resources, since cloning of mammals is an expensive venture. The reconstruction of many oocytes for some species can involve large amounts of labor, albeit repetitive in nature, and high costs for infrastructure and personnel.

82. Based on my experience in cloning mammals, one way to maximize one's limited resources for cloning mammals is to improve the efficiency of the cloning process. Such improvements in cloning efficiency have been widely reported in the scientific literature, including many articles referenced herein. However, these improvements in efficiency are not strictly required for successful somatic cell nuclear

transfer; an alternative approach is to simply increase the overall number of reconstructed embryos transferred to recipients.

83. In my experience, successful clonings of previously-reported cloned species using increased numbers of reconstructed oocytes are not usually reported in publications, because they are not "publication worthy." These clonings are simply repeating what was already known.

84. Westhusin et al. (2001) states that "access to large numbers of oocytes at relatively low cost also provides the opportunity to carry out numerous attempts at cloning. Therefore, even if the overall efficiency is low, chances are given enough trial and enough embryos transferred, a clone of most any cow or bull could be produced." (At 37, first full paragraph.)

85. I understand that Westhusin is concluding that the successful cloning of a particular cow is virtually guaranteed by reconstructing a sufficient number of nuclear transfer embryos. That is, Westhusin is acknowledging that differences in efficiency can be compensated by reconstructing and transferring more nuclear transfer embryos.

86. Based on my experience cloning mammals, I agree with this statement, and believe that it applies to all species of mammals.

87. Westhusin et al. (2001) states: "Pigs represent an excellent example for pointing out that assisted reproduction technologies and techniques for nuclear transfer don't directly apply from one species to another." (At 39, second full paragraph.)

88. Westhusin does not explain what is meant by this passage. Westhusin may be referring to the fact that efficiency of cloning is relatively low in pigs. Westhusin

may also be referring to the fact that more than pigs have a particular requirement for four or more viable embryos to maintain pregnancy.

89. I have read an article by E. Pennisi and G. Vogel entitled "Clones: A hard Act to Follow" in Science 288:1722-1727 (2000)("Pennisi et al. (2000)"), a copy of which is attached.

90. Pennisi et al. (2000) indicates that several groups had difficulties cloning pigs, and indicates that the problem with cloning pigs was that "until 1998, few scientists had tried to work with immature pig eggs or to grow embryos in the lab. Pigs differ from cows and sheep in that they are born in litters, and unless there are at least four viable fetuses in the womb, the pregnancy fails. That means that a day's work has to yield at least several viable embryos if the cloning experiment is to have any chance of success." (At 1724-1725.)

91. In my opinion, Pennisi does not indicate that anything more than routine experimentation was required to clone pigs. Based on the Campbell '233 application, Pennisi et al. (2000), Polejaeva et al. (2000), Onishi et al. (2000), Betthauser et al. (2000), Lai et al. (2002), and my own experiences cloning pigs, it is my opinion that, once sufficient viable reconstructed nuclear transfer embryos were implanted in surrogate pigs, pig cloning was successful for many different groups. I would have understood in February of 1997, based on Polge et al. (1966), that somatic cell cloning of pigs by nuclear transfer would require having at least 4 viable NT embryos to maintain pregnancy, and that failure to reconstruct sufficient nuclear transfer embryos would likely lead to failure. In my opinion, the failure in the ability of some laboratories to clone pigs between 1997 and 2000 was likely due to these laboratories

reconstructing and/or implanting insufficient embryos to maintain pregnancy. In my opinion, these failures could have been remedied by reconstructing and transferring more nuclear transfer embryos to each surrogate pig.

DIFFERENCES BETWEEN A CLONE AND ITS DONOR MAMMAL

92. I have been asked to describe differences between a live-born clone of a pre-existing, non-embryonic, donor mammal and the donor mammal itself.

93. I understand that such a clone is currently claimed in the 'Campbell '233 application. I will refer to such a clone as "Applicant's clone."

94. Based on my experience cloning mammals, Applicant's clone is not made by nature.

95. Based on my experience cloning mammals, Applicant's clone can only be made by human intervention.

96. Based on my experience cloning mammals, Applicant's clone is not an exact copy of its donor mammal.

97. Based on my experience in mammalian reproduction, environmental factors would generate differences between Applicant's clone and its donor mammal.

98. Based on my experience cloning mammals, Applicant's clone could not exist before it was made.

99. Based on my experience cloning mammals, Applicant's clone occupies different space and time than its donor mammal, and is a time-delayed, inexact copy of its donor mammal. The clone contains the same genetic complement as its donor mammal, but is not an exact copy due to environmental differences during development.

100. The ability of Applicant's clone to exist at a time later than its donor mammal, but have the same genetic complement, is a markedly different characteristic from any mammal found in nature.

101. Based on my experience in mammalian reproduction, no mammal found in nature is a time-delayed copy of either of its parents.

102. Based on my experience cloning mammals, Applicant's clone provides an alternative, time-delayed source of nuclear genomic material of its donor mammal.

103. Based on my experience cloning mammals, this feature of Applicant's clone does not depend on the continued existence of the donor mammal.

104. Consequently, Applicant's clone can provide an alternative source of nuclear genomic material of its donor mammal, even if the donor mammal is dead.

105. The time delay of Applicant's clone allows the preservation of the genomic material of a particular mammal beyond the normal lifespan of that mammal.

106. Normally, when a mammal dies, its particular genomic composition is lost. Its progeny only contain one-half of each of its two parents' genomic material. The genomic material of one parent is inextricably scrambled together with the other parent's genomic material to create the progeny.

107. Based on my experience cloning mammals, Applicant's clone avoids this permanent loss of a particular genomic composition.

108. Thus, Applicant's clone provides the potential for the continuation of a particular genomic composition in a way that never occurs in nature.

109. Based on my experience cloning mammals, this cannot be considered a trivial difference as compared to a clone's donor mammal, which does not have this potential.

110. Moreover, Applicant's clone requires two animals, namely, a pre-existing, non-embryonic, donor mammal and a clone of that donor mammal. Based on my experience in mammalian reproduction, nature never makes such a pair of mammals.

DIFFERENCES BETWEEN SCNT CLONES AND EMBRYONIC CLONES

111. I have been asked to describe differences between a live-born clone of a pre-existing, non-embryonic, donor mammal and a clone made by the embryonic cloning procedures of a number of articles, which I will specify below.

112. By "embryonic cloning procedures," I am referring to a process of cloning using nuclear transfer starting with an embryo as the nuclear donor. In this procedure, the embryo is destroyed in the process at a time when its genetic potential is unknown.

113. I have read an article by M. Sims and N.L. First entitled "Production of Calves by Transfer of Nuclei from Cultured Inner Cell Mass Cells" in *Proc. Nat. Acad. Sci. USA* 91:6143-6147 (1994)("Sims et al. (1994)"), a copy of which is attached hereto.

114. Sims et al. (1994) reports the production of calves by nuclear transfer using cultured inner cell mass cells. (At 6143, Abstract.)

115. In Sims et al. (1994), the inner cell mass cells were generated by performing immunosurgery on *in vitro* cultured embryos. (At 6143-6144, bridging paragraph.)

116. In Sims et al. (1994), the *in vitro* cultured embryos were generated by *in vitro* fertilizing oocytes with sperm. (*Id.*)

117. Thus, sexual reproduction was used to generate the embryos used to generate the cells for the nuclear transfer procedures of Sims et al. (1994).

118. The calves of Sims et al. (1994) were derived using cells derived from embryos as the nuclear donor cells. (At 6143, Abstract.)

119. These embryos were destroyed during the procedure used to make the donor cells for nuclear transfer.

120. I have read an article by K.J. McLaughlin L. Davies, and R.F. Seamark entitled "In vitro Embryo Culture in the Production of Identical Merino Lambs by Nuclear Transfer" in *Repod. Fertil. Dev.* 2, 619-622 (1990)("McLaughlin et al. (1990)"), a copy of which is attached hereto.

121. McLaughlin et al. (1990) reports the production of lambs by nuclear transfer using cells from embryos at the 8- to 16-cell stage. (At 619, Abstract.)

122. In McLaughlin et al. (1990), the embryos were generated by artificial insemination with sperm. (At 619, last paragraph.)

123. Thus, sexual reproduction was used to generate the embryos used to generate the cells for the nuclear transfer procedures of McLaughlin et al. (1990).

124. The lambs of McLaughlin et al. (1990) were all derived using cells derived from embryos as the nuclear donor.

125. These embryos were destroyed during the procedure used to make the cells for nuclear transfer.

126. I have read an article by R.S. Prather, M.M. Sims, and N.L. First entitled "Nuclear Transplantation in Early Pig Embryos" in *Biology of Reproduction* 41, 414-418 (1989)("Prather et al. (1989)"), a copy of which is attached hereto.

127. Prather et al. (1989) reports the production of a pig by nuclear transfer using a cell from an embryo at the 4-cell stage. (At 414, Abstract.)

128. In Prather et al. (1989), the embryo was generated by breeding pigs. (At 414-415, bridging a.)

129. Thus, sexual reproduction was used to generate the embryos used to generate the cells for the nuclear transfer procedures of Prather et al. (1989).

130. The pig of Prather et al. (1989) was derived using a cell derived from an embryo as the nuclear donor.

131. The embryo was destroyed during the procedure used to make the cells for nuclear transfer.

132. I have read an article by Z. Yong, W. Jianchen, Q. Jufen, and H. Zhiming entitled "Nuclear Transplantation in Goats" in *Theriogenology* 35, 299 (1991)("Yong et al. (1991)"), a copy of which is attached hereto.

133. Yong et al. (1991) reports the production of goats by nuclear transfer using cells from embryos at the 4- to 32-cell cell stage. (At 299, Table.)

134. In Yong et al. (1991), the embryos were generated from pregnant goats. (*Id.*)

135. Thus, sexual reproduction was used to generate the embryos used to generate the cells for the nuclear transfer procedures of Yong et al. (1991).

136. The goats of Yong et al. (1991) were all derived using cells derived from embryos as the nuclear donor.

137. These embryos were destroyed during the procedure used to make the cells for nuclear transfer.

138. I have read an article by H.-T. Cheong, Y. Takahashi, and H. Kanagawa entitled "Birth of Mice after Transplantation of early Cell-Cycle-Stage Embryonic Nuclei into Enucleated Oocytes" in *Biology of Reproduction* 48, 958-963 (1993)("Cheong et al. (1993)"), a copy of which is attached hereto.

139. Cheong et al. (1993) reports the production of mice by nuclear transfer using cells from embryos at the 2- to 8-cell cell stage. (At 619, Abstract.)

140. In Cheong et al. (1993), the embryos were generated from mated females. (At 958, last paragraph.)

141. Thus, sexual reproduction was used to generate the embryos used to generate the cells for the nuclear transfer procedures of Cheong et al. (1993).

142. The mice of Cheong et al. (1993) were all derived using cells derived from embryos as the nuclear donor.

143. These embryos were destroyed during the procedure used to make the cells for nuclear transfer.

144. I have read an article by X. Yang, S Jiang, A. Kovacs, and R. Foote entitled "Nuclear Totipotency of Cultured Rabbit Morulae to Support Full-Term development Following Nuclear Transfer" in *Biology of Reproduction* 47, 636-643 (1992)("Yang et al. (1992)"), a copy of which is attached hereto.

145. Yang et al. (1992) reports the production of rabbits by nuclear transfer using cells from embryos at the 32- to 64-cell cell stage. (At 636, Abstract.)

146. In Yang et al. (1992), the embryos were generated by artificial insemination with sperm. (At 636, col. 2, first paragraph.)

147. Thus, sexual reproduction was used to generate the embryos used to generate the cells for the nuclear transfer procedures of Yang et al. (1992).

148. The rabbits of Yang et al. (1992) were all derived using cells derived from embryos as the nuclear donor.

149. These embryos were destroyed during the procedure used to make the cells for nuclear transfer.

150. Thus, Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) each describes clones made by embryonic cloning procedures.

151. The embryos used as the nuclear donors in the embryonic cloning procedures of Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) were generated by normal sexual reproduction.

152. Thus, the embryos used as the nuclear donors in the embryonic cloning procedures of Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) were not identical to either of its parents.

153. The embryos used as the nuclear donors in the embryonic cloning procedures of Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) were destroyed during the embryonic cloning procedures.

154. Thus, the embryos used as the nuclear donors in the embryonic cloning procedures of Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) were never "non-embryonic."

155. The non-embryonic parental mammals in Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) would have been the two parents of each of the embryos used as the nuclear donors in the embryonic cloning procedures.

156. The clones made by Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) were not clones of these mammals, since sexual reproduction was used to generate the embryos used in the embryonic cloning procedures.

157. Based on my experience in cloning mammals, the genetic complement of the clones generated by the embryonic cloning procedures of Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) would have been a mixture of the genetic complement of it two parents, and would not have had the same genetic complement as either of the parents.

158. Consequently, the clones generated by the embryonic cloning procedures of Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) could not be a live-born clone of a pre-existing, ***non-embryonic***, donor mammal.

159. Rather, the clones generated by the embryonic cloning procedures of Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991),

Cheong et al. (1993), and Yang et al. (1992) were live-born clones of a pre-existing, donor embryo, which itself was the product of sexual reproduction.

160. A live-born clone of a pre-existing, non-embryonic, donor mammal is a time-delayed, inexact copy of a non-embryonic mammal. Based on my experience in mammalian cloning, Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) did not generate such an animal.

161. A live-born clone of a pre-existing, non-embryonic, donor mammal requires two animals, namely, a pre-existing, non-embryonic, donor mammal and a clone of that donor mammal. Based on my experience in mammalian cloning, Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) did not generate such a pair of mammals.

162. In none of Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) did a pre-existing, non-embryonic, donor mammal and a clone of that donor mammal exist.

163. Moreover, the embryonic cloning procedures of Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) preclude the coexistence of the clone and the donor.

164. This is due to fact that, in the embryonic cloning procedures of Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992), the embryonic donor was destroyed during the generation of the clone.

165. In contrast, a live-born clone of a pre-existing, ***non-embryonic***, donor mammal could coexist with its non-embryonic donor since the generation of such a clone does not require destruction of the donor mammal in generating the clone.

166. In my opinion, a live-born clone of a pre-existing, non-embryonic, donor mammal is not disclosed in Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), or Yang et al. (1992).

167. Prior to the birth of Dolly, as reported in Wilmut et al., Viable offspring derived from fetal and adult mammalian cells, *Nature* 385:810-13 (1997), I would have expected that the generation of a live-born clone of a pre-existing, non-embryonic, donor mammal was not possible.

168. Although Sims et al. (1994), McLaughlin et al. (1990), Prather et al. (1989), Yong et al. (1991), Cheong et al. (1993), and Yang et al. (1992) demonstrated that cloned mammals could be made using embryonic nuclear donor cells, I did not expect that it was possible, prior to the birth of Dolly, to generate a cloned mammal using non-embryonic nuclear donor cells.

169. The undersigned further declares that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing therefrom.

Dated: Mar. 28, 2008, By: _____
Irina A. Polejaeva, Ph.D.

MINIREVIEW

Nuclear Transplantation as a Method for Cloning Embryos (43112)

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Historical Perspective

The concept of transferring nuclei from multicell stage embryos to enucleated oocytes was first proposed by Spemann in 1938 (1). Spemann was a developmental biologist whose concern at the time was that of nuclear equivalence. The idea of the basic composition of the nuclei changing during early embryo development gave rise to the theory that nuclei from different tissues were not equal. To answer this question, Spemann proposed an experiment to transfer nuclei from progressively more advanced embryos to enucleated, activated oocytes. The conclusion of the experiment would be determined when inequivalence was achieved and the transferred nucleus would not support development to a mature adult. Results from Spemann's experiment were first reported in 1952 by Briggs and King (2) in the amphibian *Rana pipiens*. They (2) showed that nuclei from preblastula stage embryos could promote development to the blastula stage. In addition, they alluded to the observation that nuclei from beyond the blastula stage had a lesser probability of promoting development after nuclear transfer.

The spin-off of such a project is the possibility of cloning. Since all of the nuclei of the early embryo are presumed to be identical (all containing the same complement of genetic material), subsequent transfer to enucleated, activated oocytes with development to term should result in genetically identical individuals (the reality of identical individuals will be discussed later). This procedure, in combination with serial nuclear transfer (i.e., growing the first nuclear transfer embryos to the donor cell stage and then repeating the procedures) could, theoretically, result in an unlimited number of identical individuals. The possibility of this in mammals became much more likely after the successes of such procedures were reported in 1986 by Willadsen (3) and Prather *et al.* (4) in sheep and cattle, respectively.

In this review I hope to provide information on the procedures for nuclear transfer, present the concept of nuclear remodeling and reprogramming, list the factors to consider regarding the similarity of nuclear transfer embryos, and what will be necessary for commercialization of such technology.

Nuclear Transfer Procedures

The procedures for nuclear transfer are basically the same as those described by Briggs and King (2) for amphibians and adapted to mammals by McGrath and Solter (5). First, a group of recipient unfertilized oocytes is enucleated. This is accomplished after treating the oocytes with microfilament- and microtubule-inhibiting drugs such as cytochalasin B and colchicine. With the disruption of the cytoskeleton, the plasma membrane is much less likely to rupture. The recipient oocyte is held in place by aspiration with a micropipette (Fig. 1A) and another micropipette is punctured through the zona pellucida. The polar body and metaphase chromosomes are aspirated into the pipette. When the pipette is removed, the plasma membrane pinches off, forming two membrane-enclosed vesicles (one inside the pipette and one inside the zona pellucida). Originally, the aspiration was done blind, but visualization under ultraviolet light with a DNA stain permits successful enucleation every time (6). Next, the donor embryo is aspirated against the holding pipette and a single blastomere, or karyoplast, is aspirated into the transfer pipette (Fig. 1B). The transfer pipette is then inserted into the perivitelline space of the enucleated oocyte and the karyoplast is deposited (Fig. 1C). The two cells (donor karyoplast and recipient oocyte) are then allowed to regain their spherical shape (Fig. 1D). The resulting nuclear transfer embryo is placed

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between two electrodes and an electrical pulse that causes a transient breakdown of the plasma membranes is applied. When the membranes reform, small channels are created that, due to their thermodynamic instability, enlarge forming a single cell (7, 8). The electrical pulse required for cell fusion also results in the activation of the oocyte, i.e., simulation of fertilization, thus setting into motion the events necessary for early development.

Nuclear Remodeling

After the successful transfer of a karyoplast to an enucleated, activated oocyte, the transferred nucleus undergoes remodeling such that it morphologically and metabolically resembles a pronucleus. In amphibians, this is observed by an increase in diameter of the transferred nucleus (9) and the disappearance of nucleoli within the transferred nucleus (10). In mammals, where the nucleoli only undergo ultrastructural modifications during early development and not the complete disappearance as in amphibians, the nucleoli revert to their early cleavage stage morphology (11). In addition, there is significant swelling of the transferred nuclei in mice (11, 12), rabbits (13), and pigs (14). There appears to be a 1½-hr window around the time of activation of the oocyte in which chromatin can be remodeled to be similar to pronuclei (11, 12). If the nuclei are transferred outside this window, then the nuclei either condense or fail to swell. In the rabbit, the timing criteria are even more confining since optimum activation rates are achieved only during a narrow window after ovulation (15). Interestingly, when zygotes, which are outside this window, are used as recipients development does not continue to term in cattle (16) or mice (17).

Theoretically, for optimum reprogramming, the nuclei should swell to a size similar to an endogenous pronucleus. Nuclear swelling is a result of the exchange of proteins between the cytoplasm and the nucleoplasm (18, 19). This exchange of proteins is thought to be the inducer of the swelling, not a consequence of nuclear swelling (20). Since there are components in the cytoplasm that affect nuclear volume, it is important to note that the early mammalian nuclear transfer experiments used recipient oocytes from which half of the cytoplasm was removed. This likely removed half of the components within the cytoplasm that are responsible for nuclear volume and subsequent reprogramming. A 2 × 2 factorial experiment was designed to evaluate the affect of cytoplasmic volume on nuclear swelling. Nuclear size was measured in embryos resulting from the transfer of an intact or half blastomere to either an intact or half oocyte. Neither the removal of half of the cytoplasm from the recipient oocyte, nor the transfer of half of a blastomere decreased the degree of swelling observed of the transferred nucleus (14). However, it is not yet known if these parameters are related to subsequent development.

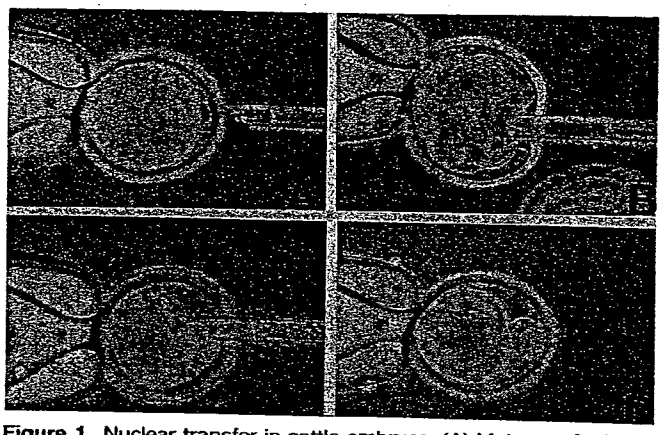

Figure 1. Nuclear transfer in cattle embryos. (A) Mature unfertilized oocyte (first polar body, a result of the completion of meiotic metaphase I, is denoted by the arrow). (B) Aspiration of blastomere containing donor nucleus. (C) Transfer of blastomere to enucleated oocyte. Arrow indicates the donor nucleus within the transfer pipette. (D) After transfer of donor blastomere, arrow indicates donor blastomere (original magnification ×400; diameter of the zona pellucida is about 150 μm). Reprinted with permission from Prather *et al.* (40).

In amphibians, after nuclear transfer, there is a shift of protein from the oocyte cytoplasm into the transferred nucleus and a shift of protein from the nucleus into the cytoplasm. This selective exchange of proteins between the nucleus and cytoplasm does not appear to be limited by the nuclear envelope, but by selective binding sites within the nucleus (21). In *R. pipiens*; nonhistone [^{3}H]tryptophan-containing proteins leave transferred endodermal nuclei, but [^{3}H]-lysine-containing proteins remain in the nucleus (18, 19). Synchronous with the release of labeled proteins is the acquisition of both acid and basic proteins by the nucleus (20).

In mammals, there is at least one suggestion of a similar exchange of nuclear lamins between transferred nuclei and cytoplasm. Nuclear lamins are a class of intermediate filaments that line the inner nuclear envelope and polymerize and depolymerize with the cell cycle. In the mouse, cow, and pig the A/C nuclear lamin epitope (antibody J9) becomes undetectable after the transition to zygotic control of development. In both the mouse and pig, if nuclei beyond the transition to zygotic control of development are transferred to enucleated activated oocytes, then the transferred nucleus acquires the lamin A/C epitope (Fig. 2) (22, 23). This is suggestive of acquisition of the lamin A/C from the cytoplasm of the oocyte. However, in the mouse, if nuclei are transferred to either an intact or enucleated zygote, then the transferred nuclei do not acquire the epitope (23). The inability of nuclear lamins to exchange between interphase cells has also been demonstrated in tissue culture (24). I hypothesize that the

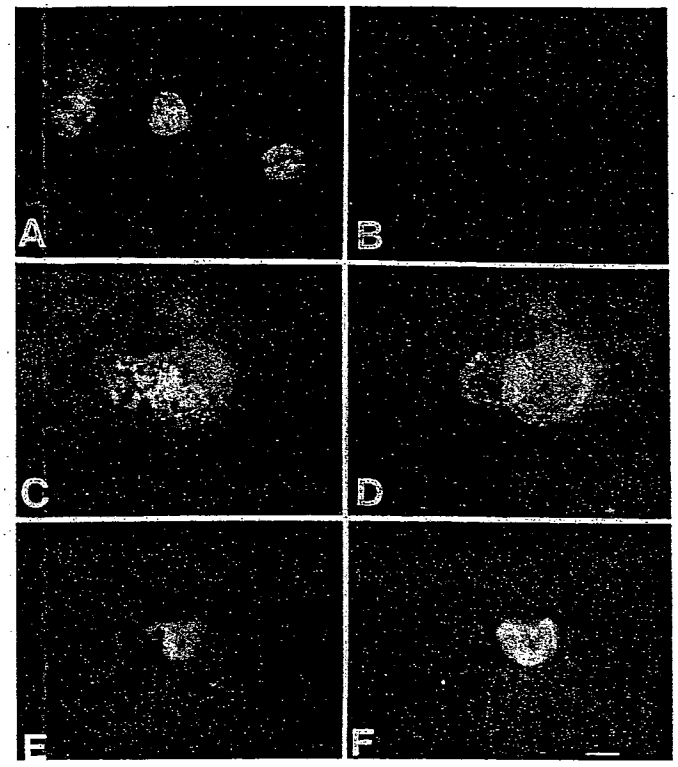

Figure 2. Nuclear lamin epitope after transfer of a 16-cell stage porcine nucleus to an activated, enucleated meiotic metaphase II arrested oocyte. DNA (Hoechst 33258) in cells from a 16-cell stage embryo (A), pronuclear stage egg (B), and activated egg after nuclear transfer (nucleus from 16-cell embryo in A; E), and corresponding lamin A/C (antibody J9) reactivity (B, D, and F, respectively). Note the absence of lamin A/C reactivity in the 16-cell stage blastomeres, whereas after transfer to an activated enucleated meiotic metaphase II oocyte and 2 hr of culture, the nucleus acquires the antigen. B, D, and E were photographed and developed under identical conditions. Cells in A, B, E, and F were mounted and stained on the same coverslip (bar = 10 μm). Reprinted with permission from Prather *et al.* (22).

inability to acquire the lamin A/C epitope is a result of the sequestering of cytoplasmic lamin A/C by the endogenous pronuclei. Thus, when an interphase cell is used as a recipient, the nuclei do not have access to the A/C lamin proteins within the cell. However, when a metaphase cell is the recipient, then the nuclear lamin proteins are dispersed in the cytoplasm and available to be incorporated into the nuclear envelope of the transferred nucleus.

Additional remodeling that is necessary before development can continue is a synchronization of the DNA synthetic phase of the donor and recipient cells. Nuclei that are in G_1 when transferred to an activated, enucleated oocyte need to complete DNA synthesis and progress to G_2 before the recipient cell attempts to divide. It is also important that nuclei in G_2 do not undergo DNA synthesis before mitosis, as the resulting embryo would be polyploid. The inability to complete DNA synthesis may be one of the most important factors affecting the resulting development. Most of the chromosomal abnormalities in amphibian nuclear transfer embryos can be traced to events that occur in the first cell cycle (25). The major problem in achieving synchrony between the donor and recipient cells is the fact that as a tissue differentiates the length of the cell cycle increases. These nuclei with long cell cycles, when transferred to an activated, enucleated oocyte, are required to complete DNA synthesis and prepare for another mitosis within an hour. Thus, relatively more differentiated nuclei are more likely to result in chromosomal abnormalities (26, 27), but any conclusions about differentiated cells versus less differentiated cells is confounded with the length of the cell cycle of the donor embryo.

Nuclear Reprogramming

The only indication, published to date, of reprogramming in mammals is the reprogramming of morphologic events. For example, if a cell from a 32-cell stage bovine embryo is transferred to an enucleated activated metaphase II oocyte and is not reprogrammed, the resulting embryo should attempt to form a blastocoele after one cleavage (at the 2-cell stage). If successful this would result in a trophoblastic vesicle that contains no inner cell mass, and hence no fetus. In addition, the absence of reprogramming would require that the nuclear transfer embryo be transferred to a surrogate mother than was synchronous with the donor 32-cell stage embryo. This would be true since domestic animals require close synchrony between the stage of the estrus cycle and stage of the developing embryo. If, instead, the 32-cell stage nucleus was reprogrammed to behave as a zygote, then the resulting embryo would begin compaction at the 32-cell stage, form a blastocoele at the 64-cell stage, and be transferred to a surrogate that ovulated synchronously with the nuclear transfer. Thus, a reprogrammed nucleus would retraverse the early cleavage stages followed by compaction and blastocoele formation, whereas control nonmanipulated embryos would attempt to continue their developmental sequence of events.

For a full appreciation of the biochemical reprogramming that presumably occurs after nuclear transfer in mammals, it should be understood that the early mammalian embryo does not begin producing RNA until a species-specific cell stage. Transcription can first be detected in the mouse embryo at the 2-cell stage (28), at the 4-cell stage in the pig (29) and rat (30), and at the 8- to 16-cell stage in the cow, rabbit, and sheep (31–33). Prior to this time the embryo relied upon RNA stored in the oocyte during oogenesis. Thus, for detectable biochemical reprogramming, nuclei need to be transferred at or after the transition to zygotic control of development. The most advanced nucleus to result in development to term by species is listed in Table I.

Table I. Relative Degree of Differentiation and Development after Nuclear Transfer to an Enucleated Activated Oocyte

Species	Stage of major transition to zygotic of development	Most advanced cell reported to result in term development
Pig (37)	4-Cell	4-Cell
Rabbit (13)	8- to 16-Cell	8-Cell
Cow (38–40)	8-Cell	32-Cell
Sheep (34)	8- to 16-Cell	Inner cell mass

The listing in Table I should not be interpreted to be maximum stage of development that will result in complete reprogramming, as few studies have attempted determine this parameter. The sheep should result in the most dramatic biochemical reprogramming since an inner cell-mass stage nucleus has resulted in term development (34).

In amphibians, the reprogramming is much more specifically described. Not only is there obvious morphologic reprogramming, similar to that described above, but there is also very specific biochemical reprogramming that occurs. Two of the most specific examples include the muscle-specific actin gene and the *5S*^{ooc} gene. Muscle-specific actin is produced by new transcription and translation in the differentiating myotome cells of the gastrula stage embryo. After the transfer of postgastrula stage myotome cell nuclei to enucleated activated oocytes, the production of muscle-specific actin RNA ceases. Muscle-specific actin RNA synthesis is not reinitiated until the embryo develops to the gastrula stage, and then only in the differentiating myotome cells (35). Similarly, the *5S*^{ooc} gene is translated and transcribed for a short period of time at the late blastula stage. Nuclei from beyond the blastula stage that are transferred to an enucleated activated oocyte transcribe the *5S*^{ooc} gene for a short period as the resulting embryo passes through the blastula stage (36). Thus, the biochemical reprogramming that occurs in amphibians is very precise.

Identical Individuals

Genetic Variation. Since all of the nuclei of early embryos are presumed to be identical, the offspring resulting from nuclear transfer should all have identical nuclear genetics. However, if prior to nuclear transfer some chromosomal rearrangement occurs in a single blastomere of the donor embryo, then this genetic defect would be passed to all subsequent embryos. Some possibilities of chromosomal rearrangement include DNA rearrangements (as happens in the normal differentiation of immunoglobulins) (41), gene amplification, translocations, and diminution.

A second factor to consider is the non-nuclear genetics. Cytoplasmic inheritance would be important when the source of the recipient oocytes are not known, such as when using *in vitro* matured oocytes derived from slaughterhouse animals. The main source of cytoplasmic inheritance is likely from the mitochondria. Even if the recipient eggs are derived from the same breed of livestock, it is disturbing that within a single maternal line of cattle the mitochondrial genome has been observed to change (42). Since the nucleus directs the synthesis of proteins used in the mitochondria (43), the interaction between the two may be very important.

There may be other organelles within the cytoplasm that have their own genome. One example may be the centriole. Recent evidence suggests that basal bodies have their own genome (44). Basal bodies are derived from the centriole, and thus the conclusion that centrioles may have their own DNA. It is very interesting to note that centrioles are absent in mammals during the first few cleavages after fertilization (45, 46). Since the presence or absence of the centrioles is related to the shape of the mitotic spindle (46), it is interesting to speculate what would happen to the centrioles and shape of the mitotic spindle after the transfer of an embryonic cell which had centrioles to an oocyte which has no centrioles!

Whether differences in cytoplasmic inheritance as described above are important in determining subsequent development remains to be determined. The possibility of such factors affecting development does exist and should be investigated.

Phenotypic Variation. The factors that affect phenotypic variation include environment and genetics. Since the possible genetic variation has already been discussed, this section will focus on environmental affects on development.

Phenotypic variation exists even in monozygotic twins. Since both cytoplasmic and nuclear genetics are presumed to be identical in monozygotic twins, all variation observed is due to environmental factors. Interestingly, cattle embryos split at the morula stage result in calves that are considered monozygotic twins, but they do not always look alike. Differential migration of the melanocytes results in twins that have the same basic color pattern, but are not identical. For example, one of the twins may have a red patch over its eye, while the other has a similar patch below its eye (47). Embryos that result from nuclear transfer are in different environments from the moment of transfer. Thus, the differences in epigenetic phenomena, *in vitro* environment, uterine environment, neonatal environment, and postnatal environment all have effects upon the resulting phenotype (48).

The first requirement for nuclear transplant embryos to be identical is identical genetics, and the second requirement is identical environments. It is likely that identical genetics (both cytoplasmic and nuclear) can

be evaluated and controlled; however, an identical environment is much more difficult to control.

Lest one is left with the impression that nuclear transfer embryos, or for that matter twins, are not phenotypically similar, it is necessary to end this with additional information. Above the impression has been given that nuclear transfer embryos or twins are not phenotypically identical. Using a strict definition this is true; however, for most researchers an increase in genetic uniformity would be very advantageous for reducing the number of animals needed for statistically valid results. Genetically similar animals not only have more similar growth characteristics than unrelated controls, but also have more similar behavior patterns (reviewed by Biggers (49)). Thus, from a research standpoint genetically uniform animals would be very useful. There would also be advantages for commercial livestock production as described below.

Commercialization

For the commercial livestock application of cloning by nuclear transfer, additional technologies must be developed. *In vitro* matured oocytes must be used as recipient oocytes, it must be possible to develop the resulting embryo *in vitro* to a stage that can be nonsurgically transferred to a recipient, and it must be possible to preserve the embryos by freezing. The pig is at a disadvantage as compared with the sheep and cow, since pig embryos are less tolerant to freeze preservation (50). In addition, only recently have methods been presented that result in normal *in vitro* development (51, 52) and offspring following nonsurgical embryo transfer (53).

An additional requirement for commercial application is a market. This could be developed after the uniformity of an individual clonal line is established. A company or producer could market a clonal line of embryos that would have a guaranteed birthing ease, neonatal growth rate, postweaning growth rate, behavioral characteristics, disease resistance, marbling characteristics, and size and shape of various meat cuts. As the phenotype of the clonal line is established and tested, these parameters would be defined as well as the ration and environment to achieve these parameters.

Future Direction

Future directions should focus mainly upon making the procedures more efficient, defining the impact of the genetic contributions, and defining the biochemical reprogramming that occurs to transferred nuclei. The current source of donor nuclei is from early stage embryos; however, a possible additional source of donor nuclei may be from embryonic stem cells. These cells have been isolated from mouse embryos, they can be cultured *in vitro* to large numbers, and, most important, they can be chimerized with the inner cell mass of blastocyst stage embryos. The embryonic stem cells that are incorporated into the fetus are stabile and can contribute to the germ line (reviewed by Prather *et al.* (54)). Since these are a relatively undifferentiated cell type, they are good candidates for a source of nuclei for the nuclear transfer. Since these cells can be transformed *in vitro*, lines of transgenic animals are a distinct possibility.

The clonal technology has a very bright future. Currently, the various steps in the procedure need not only to be refined, but in some cases defined. The procedures are inefficient, but they do work and it is now our opportunity to evaluate the parameters that affect development after nuclear transfer to make the overall procedure more efficient.

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Production of Cloned Calves Following Nuclear Transfer with Cultured Adult Mural Granulosa Cells[1]

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ABSTRACT

Adult somatic cell nuclear transfer was used to determine the totipotent potential of cultured mural granulosa cells, obtained from a Friesian dairy cow of high genetic merit. Nuclei were exposed to oocyte cytoplasm for prolonged periods by electrically fusing quiescent cultured cells to enucleated metaphase II cytoplasts 4–6 h before activation (fusion before activation [FBA] treatment). Additionally, some first-generation morulae were recloned by fusing blastomeres to S-phase cytoplasts. A significantly higher proportion of fused embryos developed in vitro to grade 1–2 blastocysts on Day 7 with FBA (27.5 ± 2.5%) than with recloning (13.0 ± 3.6%; $p < 0.05$). After the transfer of 100 blastocysts from the FBA treatment, survival rates on Days 60, 100, 180, and term were 45%, 21%, 17%, and 10%, respectively. Ten heifer calves were delivered by elective cesarean section; all have survived. After the transfer of 16 recloned blastocysts, embryo survival on Day 60 was 38%; however, no fetuses survived to Day 100. DNA analyses confirmed that the calves are all genetically identical to the donor cow. It is suggested that the losses throughout gestation may in part be due to placental dysfunction at specific stages. The next advance in this technology will be to introduce specific genetic modifications of biomedical or agricultural interest.

INTRODUCTION

There is intense scientific interest in the field of somatic cell nuclear transfer, principally to enable both the multiplication of elite livestock and the engineering of transgenic animals, for various agricultural and biomedical purposes. The realization of these applications involves the development of embryo and cell manipulation techniques that facilitate the totipotent potential of cultured and/or differentiated cell nuclei to be expressed.

The successful production of offspring derived after nuclear transfer depends upon a wide variety of factors. One factor of importance is appropriate cell cycle coordination. Although there are no definitive reports comparing the effects of cell cycle synchrony between donor nuclei from cultured somatic cells and recipient cytoplasts, there currently appear to be at least two methods that are successful in yielding viable cloned offspring. The first uses donor cells in a quiescent state, in which the cells are presumed to have exited the normal cell cycle and have arrested in what is termed G0. This may be induced in cultured cells by, for example, serum starvation [1, 2] or by using cells that are naturally arrested in this state directly from the animal [3, 4]. It is possible that the reduction in transcriptional activity and chromatin modification associated with cells in G0 [5] may facilitate the reprogramming of nuclei following exposure to oocyte cytoplasmic factors, enabling normal development to occur in some instances [1, 2]. The failure of earlier studies in amphibians to generate viable clones from adults after the use of G0 cells (e.g., [6–8]) may have been due to other limiting facets of the nuclear transfer technique and/or the greater difficulty (or impossibility) to reprogram the nuclei of some terminally differentiated cell types. The latter phenomenon may be the case with Sertoli and neuronal cells in the mouse [3].

The second method that appears successful in somatic cell nuclear transfer involves exploiting the various factors present in the cytoplasm of the metaphase-arrested oocyte that may facilitate the remodeling and reprogramming of somatic cell nuclei [9]. In addition, prolonged exposure to this cytoplasmic environment may aid this process further and appears to have conferred nuclear totipotency to nonquiescent cells [10]. This prolonged cytoplasmic exposure may be achieved by fusing cells before the activation of the reconstructed embryo [9, 11–13]. However, for it to be successful and to avoid chromosomal damage and abnormal ploidy in the resulting embryos, the cell cycle stage of the donor nucleus must be compatible with the high levels of maturation-promoting factor present in the metaphase II (MII) oocyte [14, 15]. For these reasons, only nuclei that have a diploid (2C) DNA content (that is, in either G1 [16] or G0 of the cell cycle [1–3]), or in metaphase [17]) are compatible with nuclear transplantation to enucleated MII oocytes. Although no effect of prolonged exposure of quiescent cells to the MII cytoplasm has been observed in sheep [1], effects have been seen in other species. With unsynchronized bovine cultures of undifferentiated embryonic cells, in which the majority of cells were reported to be in G1, exposure to MII cytoplasm for 4 h before activation significantly increased development to blastocyst, compared to embryos reconstructed by either simultaneous fusion and activation, or with preactivated cytoplasts [18]. Although fetal development did not proceed beyond Day 55 in this study [18], the strategy of fusion before activation has resulted in viable cloned calves using fetal fibroblasts in another study, in which once again the majority of cells were reported to have been in G1 [10]. With quiescent bovine cells derived from either the fetus [12] or adult [13], embryo development was also significantly increased by prolonging the period of exposure of the nucleus to the MII cytoplasm before activation. Similarly in the mouse, embryo and fetal development were both improved with exposure of cumulus cells in G1/G0 to MII cytoplasm for 1–6 h [3].

Although it is now clearly possible to produce cloned offspring from differentiated mammalian cells after nuclear transfer, the overall success rate is currently low in the above-mentioned studies (0.4–1.8%). Furthermore, in most studies, the exact stage of the cell cycle of the successful donor cells, which ultimately yielded the viable cloned offspring, remains uncertain.

The principal objective of the nuclear transfer studies

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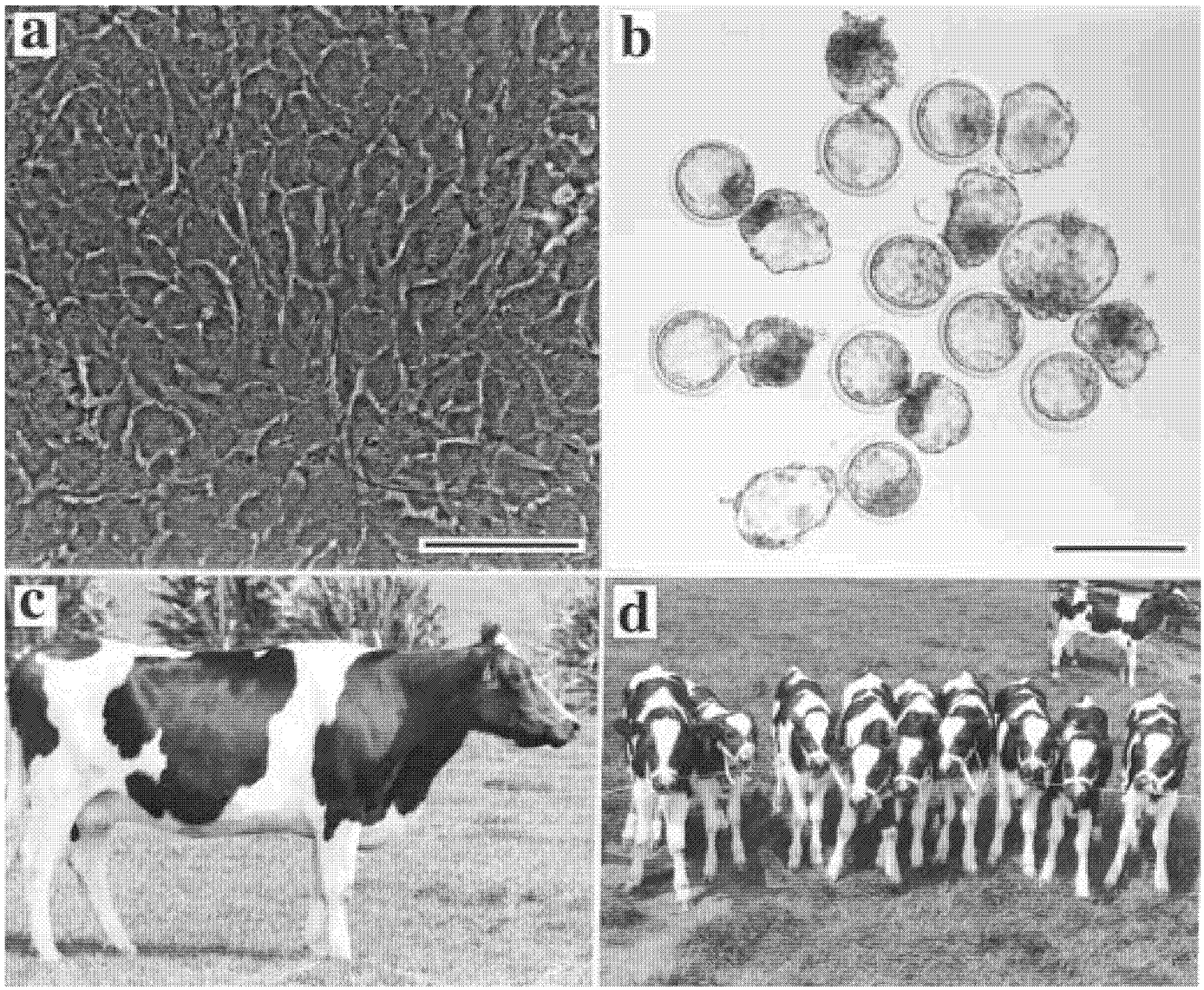

FIG. 1. Demonstration of the totipotency of adult bovine mural granulosa cells after nuclear transfer. **a**) Morphology of the EFC mural granulosa cells at passage five. Bar = 100 μm. **b**) Hatching blastocysts produced seven days postfusion from the FBA treatment with EFC cells. Bar = 200 μm. **c**) The Friesian donor cow. **d**) Ten cloned calves genetically identical to the cow shown in **c.** Insert shows one calf recovering from a broken leg at the time of submission.

described here was to determine the nuclear totipotency of cultured adult bovine mural granulosa cells. We also investigated the effect on embryo and fetal development of prolonged exposure of the transferred nucleus to the oocyte cytoplasm by either fusing quiescent granulosa cells before activation or by recloning the first-generation nuclear transfer embryos. Recloning provides an additional method of allowing a longer period for nuclear reprogramming to occur and has improved development in amphibians [9]. During the course of these experiments, we have established the efficiency of cloning adult females using mural granulosa cells and have begun to identify the important areas of future research needed to improve the success rates beyond the current 10% embryo survival to term reported here.

MATERIALS AND METHODS

Isolation of Mural Granulosa Cells

A primary cell line (EFC) was established from mural granulosa cells collected by aspirating the ovarian antral follicles (3–10 mm in diameter) from a three-year-old Friesian dairy cow of high genetic merit (Fig. 1c), using an ultrasound-guided, transvaginal probe [19]. The collected cells were centrifuged and washed once in culture medium before seeding onto a four-well tissue culture plate (Nunclon, Roskilde, The Netherlands). The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM)/F12 medium (Life Technologies, Auckland, New Zealand) supplemented with 10% v:v fetal calf serum (FCS; Life Technologies) and sodium pyruvate to a final concentration of 1 mM. During the first seven days, penicillin, streptomycin, and amphotericin B were added. The cell line was routinely passaged using an enzymatic solution (TEG) comprising 0.25% w:v trypsin (porcine pancreas; Life Technologies) and 0.04% w:v EGTA (Sigma, St. Louis, MO) for 7 min at 39°C. Small aliquots of early-passage EFC cells were frozen in 10% dimethyl sulfoxide (BDH, Poole, Dorset, England). The average cell population doubling time was 42.0 ± 1.1 h and the cells were maintained for at least nine passages in culture, representing approximately a total of

15 cell population doublings. The cell morphology is illustrated in Figure 1a. All cells used for nuclear transfer in these experiments had been previously frozen and thawed.

In Vitro Maturation of Oocytes

Slaughterhouse ovaries were collected from mature cows, placed in saline (30°C), and transported within 2 h to the laboratory. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3–6-mm follicles using an 18-gauge needle and negative pressure (40–50 mm Hg). COCs were collected into HEPES-buffered tissue culture medium 199 (H199; Life Technologies) supplemented with 50 μg/ml heparin (Sigma) and 0.4% w:v BSA (Immuno-Chemical Products [ICP], Auckland, New Zealand). Before in vitro maturation, only those COCs with a compact, nonatretic cumulus oophorus-corona radiata and a homogenous ooplasm were selected. They were washed twice in H199 medium + 10% FCS (Life Technologies) before being washed once in bicarbonate-buffered tissue culture medium 199 + 10% FCS. Ten COCs were transferred in 10 μl of this medium and placed into a 40-μl drop of maturation medium in 5-cm Petri dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) overlaid with paraffin oil (Squibb, Princeton, NJ). The maturation medium comprised tissue culture medium 199 supplemented with 10% FCS, 10 μg/ml ovine FSH (Ovagen; ICP), 1 μg/ml ovine LH (ICP), 1 μg/ml estradiol (Sigma), and 0.1 mM cysteamine (Sigma) [20]. Microdrop dishes were cultured at 39°C in a humidified 5% CO_2 in air atmosphere for 20 h. After maturation, the cumulus-corona was totally removed by vortexing COCs in 0.1% hyaluronidase (from bovine testis; Sigma) in HEPES-buffered synthetic oviduct fluid (HSOF) [21] for 3 min; this was followed by three washes in HSOF + 10% FCS.

Nuclear Transfer with Granulosa Cells

Media. With embryos that were reconstructed by fusing donor cells and MII cytoplasts before activation (fusion before activation [FBA] treatment), matured oocytes, cytoplasts, and reconstructed embryos were either held or manipulated in HSOF- or SOF-based medium (as appropriate) without calcium for the period following maturation and until 30 min before activation. After this point, calcium was present in all media formulations used.

Enucleation. Oocytes matured for 20–22 h were enucleated with a 30-μm (external diameter) glass pipette, by aspirating the first polar body and the MII plate in a small volume of surrounding cytoplasm. The oocytes were previously stained in HSOF medium containing 10% FCS, 5 μg/ml Hoechst 33342, and 7.5 μg/ml cytochalasin B (Sigma) for 20 min. Enucleation was confirmed by visualizing the karyoplast, while still inside the pipette, under ultraviolet light. After enucleation, the resulting cytoplasts were washed extensively in HSOF + 10% FCS and were held in this medium until injection of donor cells.

Preparation of cells. Donor cells were used for nuclear transfer between passages three and eight of culture. EFC cells were induced to enter a period of quiescence (presumptive G0) by serum deprivation [1]. One day after routine passage, the culture medium was aspirated, and the cells were washed three times with fresh changes of PBS before fresh culture medium containing only 0.5% FCS was added. The cells were returned to culture for a further 8–18 days before they were used for nuclear transfer. Immediately before injection, a single cell suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted and resuspended in HSOF + 0.5% FCS and remained in this medium until injection.

Microinjection. Recipient cytoplasts were dehydrated in HSOF containing 10% FCS and 5% sucrose. This medium was also used as the micro-manipulation medium. A 35-μm pipette (external diameter) containing the donor cell was introduced through the same slit in the zona pellucida as made during enucleation, and the cell was wedged between the zona and the cytoplast membrane to facilitate close membrane contact for subsequent fusion. After injection, the reconstructed embryos were rehydrated in two steps; first in HSOF containing 10% FCS and 2.5% sucrose for 5 min and then in HSOF + 10% FCS until fusion.

Cell fusion. Reconstructed embryos in the FBA group were electrically fused at 24 h poststart of maturation (hpm) in buffer comprising 0.3 M mannitol, 0.5 mM HEPES, and 0.05% fatty acid-free (FAF) BSA (Sigma) with 0.05 mM calcium and 0.1 mM magnesium. Fusion was performed at room temperature, in a chamber with two stainless steel electrodes 500 μm apart overlaid with fusion buffer. The reconstructed embryos were manually aligned with a fine, mouth-controlled Pasteur pipette, so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. Cell fusion was induced with two DC pulses of 2.25 kV/cm for 15 μsec each, delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA). After the electrical stimulus, the reconstructed embryos were washed in HSOF + 10% FCS. They were then checked for fusion by microscopic examination.

Activation. After fusion, FBA embryos were cultured for a period of 4–6 h in SOF + 10% FCS before chemical activation. Thirty minutes before activation, fused embryos were washed and held in HSOF (containing calcium) + 1 mg/ml FAF BSA. Activation was induced by incubation in 30-μl drops of 5 μM ionomycin (Sigma) in HSOF + 1 mg/ml FAF BSA for 4 min at 37°C. Embryos were then extensively washed in HSOF + 30 mg/ml FAF BSA for 5 min before culture for 4 h in 2 mM 6-dimethylaminopurine (6-DMAP; Sigma) in SOF + 10% FCS.

Recloning: Nuclear Transfer with Embryonic Blastomeres

Unlike the FBA treatment, all medium formulations contained calcium. After enucleation, cytoplasts were preactivated at 24 hpm with ionomycin and 6-DMAP, as described above. Donor blastomeres were prepared from compacting morulae 5 days after the fusion of quiescent granulosa cells. Zonae pellucidae were digested using 0.5% w:v pronase (Sigma) for approximately 30 sec. Blastomeres were separated by incubation for 30 min in calcium- and magnesium-free PBS containing 7.5 μg/ml cytochalasin B, with the aid of gentle pipetting. Blastomeres were transferred to the injection chamber in a drop of medium containing cytochalasin B and were individually injected into the perivitelline space of dehydrated cytoplasts around 6–8 h after the activation stimulus. Cell fusion was induced with two DC pulses of 1.20 kV/cm for 80 μsec each in the fusion buffer described above. Successfully fused embryos were then placed into culture as described below.

In Vitro Culture of Nuclear Transfer Embryos

Embryo culture was performed in 20-μl drops of SOFaaBSA (8 mg/ml FAF BSA; Sigma) [22] overlaid with paraffin oil. Whenever possible, groups of five to six embryos were cultured together. Embryos were cultured in a

humidified modular incubator chamber (ICN Biomedicals, Aurora, OH) at 39°C in a 5% CO_2:7% O_2:88% N_2 gas mix. On Day 5, embryos were transferred to fresh 20-μl drops of SOFaaBSA + 10% charcoal-stripped FCS (csFCS) [23]. On Day 7 postfusion, development to morulae and blastocysts was recorded, and embryos were morphologically assessed using a subjective grading system based on a scale of 1–4, inclusive, representing embryos ranging from excellent to poor quality, respectively. The cell number of some embryos was determined by counting stained nuclei, using an established image analysis method described previously [24].

Oocyte Activation Controls

Oocytes matured for 20 h were stripped of cumulus cells, and those having the first polar body were selected. Before activation at the appropriate time, oocytes were held in calcium-free medium. Oocytes were activated with ionomycin at either 24 or 30 hpm and then cultured in SOF (containing calcium) + 10% FCS and 6-DMAP, as described above for nuclear transfer embryos. After a 4-h incubation, oocytes were washed and transferred to SOF (containing calcium) + 10% FCS until they were whole-mounted and fixed, between 6 and 12 h postactivation, using methods described elsewhere [25]. After staining with 1% orcein in 45% acetic acid, all oocytes were examined by phase-contrast microscopy for the presence of pronuclei formation.

As a negative control for electrical activation at 24 hpm, oocytes were exposed to the electrical stimulation used for fusion as described above. Oocytes were then cultured in SOF + 10% FCS without calcium, exactly as used for the FBA nuclear transfer embryos, but for 10–12 h. Control oocytes were then mounted and fixed, before subsequent examination of chromatin configuration to identify oocytes in either anaphase II or telophase II, or with pronuclei, as evidence of activation.

Embryo Transfer

Recipient cows were synchronized by a single 10-day CIDR-plus (InterAg, Hamilton, New Zealand) treatment. Six days after CIDR-plus insertion, each cow received 250 mg chlorprostenol (1 ml estrumate; Schering-Plough, Union, NJ). The mean onset of estrus was observed 48 h after CIDR-plus withdrawal. Embryo transfer was performed nonsurgically on Day 7 after estrus (estrus = Day 0 = day of fusion). Each cow received two blastocysts of grade 1–2 quality, transferred in HSOF + 5% csFCS medium into the uterine lumen ipsilateral to the corpus luteum.

Determination of Embryonic Survival and Calving

All cows were examined by ultrasonography (Piemed 200 scanner, with a linear 3.5–5 MHz rectal probe; Philipsweg, Maastricht, The Netherlands) on Day 60 of gestation to record fetal development. Pregnant cows were monitored by rectal palpation at regular intervals thereafter. Commencing approximately 2 wk before expected full term, pregnant cows were monitored daily by both rectal and vaginal examination to determine fetal position and cervical dilation. Parturition was induced with an injection of 20 mg dexamethasone (Dexadreson; Intervet, Boxmeer, The Netherlands) administered 17 h before cesarean section between Days 276 and 281.

Neonatal Care

Immediately after delivery, the newborn calves were weighed and transported to a room maintained at 25°C for neonatal monitoring. The calves were dried, rectal temperature was taken, oxygen therapy was provided via a face mask, and the calves were positioned in sternal recumbency. Oxygen therapy was discontinued typically after 30 min, but this depended upon an assessment of general calf demeanor and supplementary analytical data. Blood gas and electrolyte values were determined on some calves by immediately analyzing samples of anaerobic, heparinized blood obtained from the brachial artery, using an i-STAT clinical analyzer (Sensor Devices, Waukesha, WI). The time taken for the calves to stand unaided was recorded, and colostrum was offered via a nipple bottle at this point. It was ensured that calves received a volume of colostrum equivalent to 10% of their live weight in the first 10 h. After their first feed, calves were moved to a recovery room set at 18°C overnight and were then subsequently reared outdoors. All calves received either Zaquilan (Schering-Plough) or Excenel (Upjohn, Kalamazoo, MI) as a precaution against respiratory infection. Jugular venous blood samples were taken 18 h after delivery for routine biochemistry and hematology analyses, and at regular intervals thereafter.

Microsatellite Analyses

Genomic DNA was extracted from the white blood cells collected from the nuclear transfer-derived calves and recipient cows using a guanidine hydrochloride method [26]. Sixteen microsatellite markers (see *Results* for specific loci) were analyzed using methods described elsewhere [27]. The resulting microsatellite alleles for the nuclear transfer-derived calves were compared with those from the donor cow from which the mural granulosa cells were obtained, and contrasted with those from the recipient cows that carried the respective pregnancies.

Animal Ethics

This project was approved by both the AgResearch Ruakura Animal Ethics Committee and the AgResearch Ruakura Biosafety Committee.

Statistical Analyses

The proportional data for cell fusion, in vitro development of embryos, and subsequent survival following embryo transfer were all analyzed by fitting generalized linear models using binomial distributions within the GENSTAT 5 statistical package (Lawes Agricultural Trust, Rothamsted, UK). Embryo cell numbers were analyzed after log-transformation.

RESULTS

Embryo Development

The fusion of embryonic blastomeres to cytoplasts in the recloned group was significantly higher than the fusion of quiescent granulosa cells in the FBA treatment (88.7 ± 3.8% vs. 77.4 ± 2.2%, $p < 0.05$) (Table 1). Electro-fusion with granulosa cells was not affected by either the length of time the cells were in low serum (8–18 days) or passage number (3–8).

Although there was no difference in the proportion of

TABLE 1. Effect of nuclear transfer treatment on the electrical cell fusion rates and the proportion of fused embryos developing to morulae or blastocysts by Day 7.

Treatment	Fusion	Number cultured	Blastocysts (grade 1–2)	Blastocysts (grade 1–3)	Total morulae & blastocysts
FBA	77.4%[a]	552	152 (27.5%)[c]	282 (51.1%)[e]	383 (69.4%)
Recloned	88.7%[b]	146	19 (13.0%)[d]	51 (34.9%)[f]	84 (57.5%)

[ab], [cd], [ef], $p < 0.05$.

fused embryos that developed to the morula or blastocyst stage (grades 1–4) by Day 7, significantly more FBA embryos developed into both grade 1–2 and grade 1–3 blastocysts (27.5 ± 2.5%; 51.1 ± 2.2%) compared to the recloned embryos (13.0 ± 3.6%; 34.9 ± 4.0%, respectively; $p < 0.05$) (Table 1; Fig. 1b). Within the FBA treatment, there was no effect of either granulosa cell passage number or length of time in low serum, on subsequent embryo development rates.

Embryo Cell Numbers

Embryo cell number within each embryo stage and grade category was not affected by FBA or recloning treatments, nor by length of time in low serum or passage number of the granulosa cells. The average cell number for grade 1–2 and grade 3 blastocyst-stage embryos was 132 ± 14 (n = 58) and 82 ± 3 cells (n = 166; $p < 0.05$), respectively. For those embryos that had only developed to morulae or early blastocysts seven days postfusion, the average cell number (26 ± 2) was significantly less ($p < 0.001$; n = 84).

Activation of Control Oocytes

In the FBA and recloning treatments, both the reconstructed embryos and the cytoplasts, respectively, were artificially activated, using ionomycin and 6-DMAP. However, the relative age of the cytoplasm differed, with activation occurring at 24 hpm in the recloning treatment and at 30 hpm with the FBA embryos. Studies with control MII oocytes showed that the activation rate, as observed by pronuclear formation, was not different at either 24 hpm (95.9 ± 1.6%; n = 165) or 30 hpm (93.1 ± 3.4%; n = 131).

With the experimental conditions used here to achieve cell fusion at 24 hpm in the FBA treatment, only 1.0% (n = 97) of control MII oocytes were electrically activated by the stimulus.

Embryo Survival

A total of 100 grade 1–2 blastocyst-stage embryos from the FBA treatment were transferred to 50 recipient cows. Survival rates on Days 60, 100, 180, and term were 45%, 21%, 17%, and 10%, respectively. In contrast, of the 16 embryos transferred from the recloned group, 38% resulted in fetuses present at Day 60; however, none survived to Day 100 of pregnancy. In the FBA treatment, seven fetuses were lost in the third trimester, including three sets of twin pregnancies. These losses resulted from an excessive accumulation of allantoic fluid. Ten heifer calves were delivered by cesarean section between Days 276 and 281 from eight recipient cows. All 10 cloned calves have survived (Fig. 1d). After the first few hours of life, regular animal health tests showed that the calves were physiologically healthy. The calves were derived from mural granulosa cells at either passage three or five of culture. There was no effect of length of serum deprivation or passage number on embryo survival rates.

Postnatal Characteristics of Cloned Calves

The average birth weights of the four twin calves and the six singles were 30.1 ± 2.1 kg and 44.1 ± 2.1 kg, respectively (range: 26.5–51.0 kg). After cesarean section, the average time for the majority of calves (8 of 10) to stand unaided was 39 min (range 30–50 min). However, two larger calves with birth weights of 49 and 51 kg required 90 and 120 min, respectively. All calves had a strong suckling reflex and, once standing, all drank colostrum from the bottle. In the case of one calf, 1 mg epinephrine and 20 mg doxapram were administered within 5 min of delivery in order to stimulate the cardiac and respiratory systems, respectively. This calf responded well to treatment and was standing and feeding 40 min later. The average rectal temperature within 10 min of delivery was 39.7 ± 0.1°C. However, by 1 h and 8 h postdelivery, rectal temperatures had fallen to 38.9 ± 0.2°C and 38.2 ± 0.1°C, respectively. Plasma bicarbonate was generally normal within the first hour (25.7 ± 0.8 mM; n = 3); however, one calf had metabolic acidosis over this period (16 mM HCO^{3-}). Of the five calves examined, four had normal plasma glucose levels (3.6 ± 0.5 mM); however, one calf was hypoglycemic after 90 min (1.4 mM glucose) and took several hours before it stabilized to normal levels. While all the calves were viable, a number of abnormalities were noted in the placentas of four recipient cows, which included cases of enlarged umbilical vessels, edematous membranes, and greater than usual allantoic fluid volume. None of these abnormalities appeared to compromise fetal development.

Microsatellite Analyses

Microsatellite DNA analyses examining 16 loci confirm that all the calves are genetically identical to the donor cow from which the granulosa cells used for nuclear transfer were obtained. Additionally, the cloned calves are not genetically related to the respective recipient cows (Fig. 2; a–d represent microsatellite markers texan10, bms1789, bm711, and bms941, respectively). In all of the autoradiograms, lane 1 represents the donor cow and lanes 2–11 the adult cloned calves, with lanes 12–19 representing the eight recipient cows. The other microsatellite markers examined were agla232, bmc4214, bms1353, bms2614, cssm38, oarfcb20, rm216, rm327, rm737, tgla122, tgla126, and tgla227 (data not shown).

DISCUSSION

We have shown that mural granulosa cells obtained from a living adult cow can be reprogrammed by nuclear transfer and result in the production of viable cloned calves. Exposure of the quiescent nucleus to cytoplasmic factors present in the MII oocyte for 4–6 h resulted in relatively high

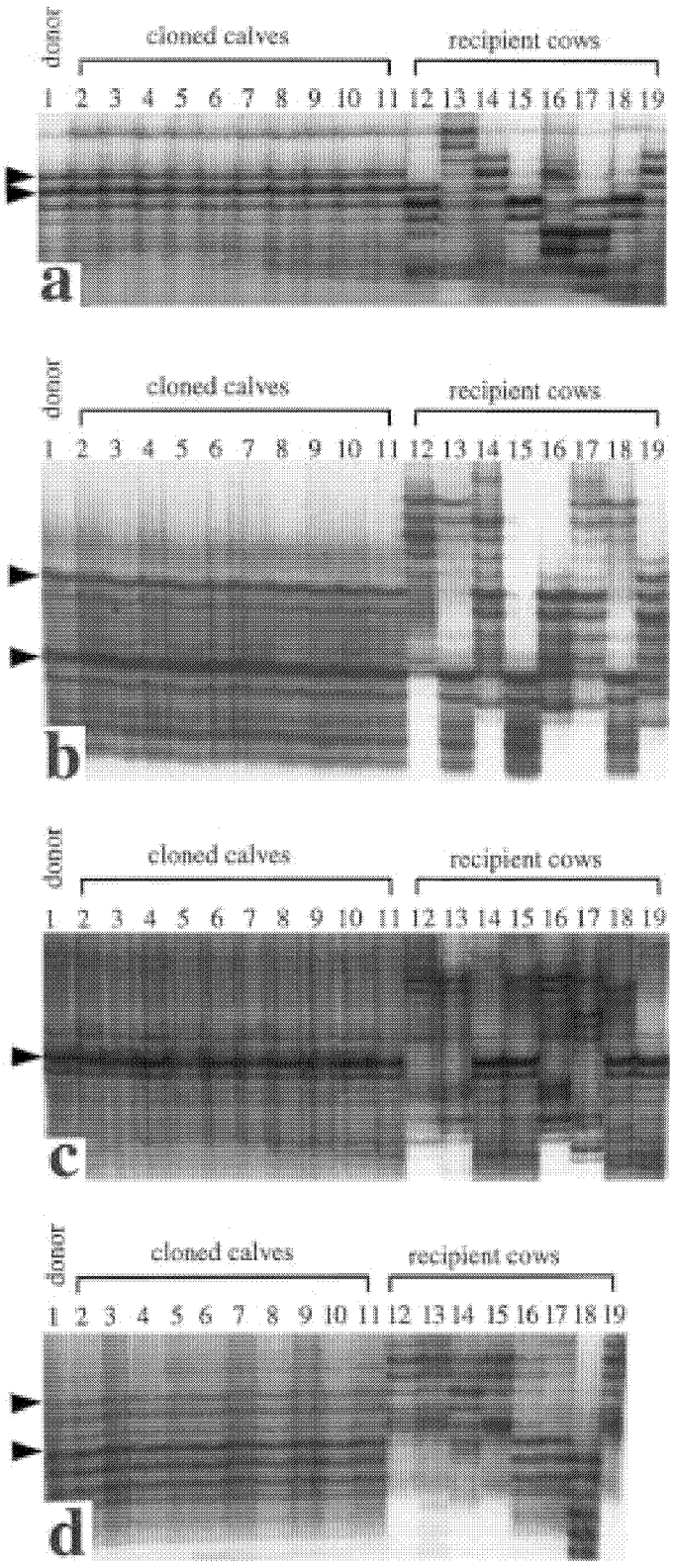

FIG. 2. Autoradiograms demonstrating the genetic origin of the cloned calves using microsatellite DNA markers texan10, bms1789, bm711, and bms941, in **a, b, c,** and **d,** respectively (see text for details).

rates of development to transferable-quality embryos in vitro (28%) and survival to term (10%). Collectively these data suggest improved reprogramming of the somatic cell nucleus. However, the technique remains limited by the substantial fetal loss that occurs throughout gestation and the abnormalities associated particularly with placentation and parturition.

It has not yet been determined which cell types from the adult animal are the most successful for somatic cell nuclear transfer. Earlier reports have shown that quiescent cells from the mammary gland in the sheep [2] and the cumulus cells surrounding the ovulated mouse oocyte [3] are both successful cell types, with overall efficiencies (in terms of live animals from successfully fused or injected cytoplasts) of 0.4% and 0.9%, respectively. In comparison, with the bovine mural granulosa cells used here for nuclear transfer, we report an overall efficiency of 2.8%, from the in vitro development and subsequent transfer of grade 1 and 2 blastocysts only (we have no data on the viability of grade 3 blastocysts). Mural granulosa cells differ in both their function and fate from cumulus granulosa cells [28]. Since the EFC cells were isolated before terminal differentiation into luteal cells, it is probable that the mural granulosa cells used here were less differentiated than expanded and mucified cumulus granulosa cells obtained from ovulated oocytes [4]. In an earlier study, bovine cumulus granulosa cells failed to produce pregnancies after nuclear transfer [29]; however, this cell type has recently resulted in the birth of adult clones in the mouse [3]. We suggest that mural granulosa cells will prove to be a suitable cell type from which to clone genetically elite cows because of the ease of repeated and noninvasive collection of cells using standard "ovum pick-up" techniques [19].

It has been shown in amphibians that the efficiency of nuclear transfer, represented as the proportion of clones developing normally, decreases as nuclei from more differentiated cell types, or more advanced developmental stages, are used [30]. Thus it had been postulated that as development proceeds, the totipotency of nuclei becomes restricted. With sheep and cattle in our laboratory, at least, this effect has not been observed. The proportion of fused embryos that develop to blastocysts (grade 1–3) from embryonic [31, 32], fetal [12], and adult (this study) cell types are 26%, 52%, and 51%, respectively. The corresponding production of viable offspring from embryos transferred in each of these categories is 5%, 11%, and 10%, respectively. Interestingly, these efficiencies with cultured somatic cells are not dissimilar to those achieved in some studies after nuclear transfer with embryonic blastomeres [33, 34]. By way of comparison, the efficiency of standard in vitro production (IVP) of bovine embryos in our laboratory is typically 40% development to blastocyst, with 39% of transferred embryos surviving to term [23]. Significantly, the quality of adult cloned embryos as determined by the cell number of grade 1 and 2 blastocysts on Day 7 is not different from that of bovine IVP embryos [23]. Thus the proportion and quality of blastocysts that develop are similar after in vitro fertilization and somatic cell nuclear transfer; however, the development to term of cloned embryos is currently only one-quarter that of IVP.

All ten nuclear transfer-derived calves reported here resulted from the transfer of embryos produced after the fusion of quiescent mural granulosa cells with enucleated MII oocytes, which were then activated to commence development 4–6 h later. We have previously demonstrated that embryo development is significantly increased by fusing quiescent donor cells with metaphase II cytoplasm before activation (FBA treatment), in comparison to simultaneous fusion and activation at either 24 or 30 hpm with either bovine fetal fibroblasts [12] or adult mural granulosa cells [13]. The prolonged exposure of transferred nuclei to oo-

cyte cytoplasmic factors possibly facilitates nuclear remodeling and reprogramming, as suggested previously [9, 35]. Improved embryo development with the FBA treatment, compared to simultaneous fusion and activation, has also been observed with unsynchronized cultures of bovine embryonic cells [18] and with mouse cumulus cells [3], in which the majority of cells were reported to be either in G1 or in a natural G0/G1 stage of the cell cycle, respectively. In addition to the effect of FBA, there may be benefits in synchronizing cells in G0, since blastocyst rates in the bovine species with unsynchronized embryonic [18] or fetal [10] cells have only been 10–12% compared to 51–52% reported with both quiescent adult and fetal cells (the present study and [12]). The lower development from unsynchronized cell populations may have been a consequence of inappropriate cell cycle coordination in approximately 40% of reconstructed embryos, as they would have received cells that were not in G1 [10, 18], and/or a consequence of inadequate nuclear reprogramming from using nonquiescent cells. It is significant to note, however, that the proportion of embryos transferred that resulted in viable calves was not different (around 10%) between MII cytoplasts reconstructed with donor cells presumed to either be in G0 ([12] and this study) and in G1 [10]. Further investigations are needed: first to verify the exact stages of the cell cycle being used in nuclear transfer studies, and then to determine the effects of various cell cycle combinations between cultured somatic cells and cytoplasts on subsequent in vitro and in vivo development.

For the FBA treatment protocol, it is important to ensure that the cytoplasts were not prematurely activated, particularly during electro-fusion. Under the experimental conditions used here, and in agreement with Stice and colleagues [18], a negligible proportion of reconstructed embryos in the FBA group would have been activated (around 1%) by the electrical stimulation employed to achieve fusion at 22–24 hpm,. Despite the presence of calcium and magnesium in the fusion buffer, added to increase fusion rates [12, 13], the young age of the cytoplasts in combination with the electrical fusion parameters used did not result in premature activation of the reconstructed embryos before exposure to ionomycin and 6-DMAP at 30 hpm.

With the introduction of the donor nucleus before activation, it is vital to control the ploidy of the reconstructed embryo after the activating stimulus is applied, in order for normal development to proceed. The relatively high rates of embryo development in the FBA group here may therefore have been in part due to the presence of 6-DMAP in the medium following exposure to ionomycin. This protein kinase inhibitor may have inhibited phosphorylations necessary for the spindle apparatus (as suggested in [36]) and therefore prevented micronuclei formation known to occur when fusion precedes activation [11]. Other researchers have used nocodazole as a microtubule inhibitor to control ploidy, but in their studies in sheep, with a quiescent embryonic cell line [1], there was no apparent benefit of FBA treatment, either in terms of embryo development or embryo survival. The lack of any treatment to prevent micronuclei formation may partly explain the poor embryo and fetal development in an earlier cloning study [29] following the transfer of a small number of embryos derived from bovine cumulus cells, presumably in G0/G1 [4] and apparently exposed to MII cytoplasm for a short period before electrical activation. In the mouse, a polar body is typically extruded after activation of MII cytoplasts reconstructed with donor nuclei [37, 38], quite unlike the situation with sheep and cattle [15]. Cytochalasin B was therefore added to control ploidy when mouse cumulus cells were fused before activation in embryos reconstructed by nuclear transfer [3].

This study also examined the recloning of embryos initially produced after nuclear transfer with quiescent granulosa cells. The aim was to investigate the effect of additional exposure of transferred nuclei to oocyte cytoplasmic factors, in order to allow a longer opportunity for nuclear reprogramming to occur by effectively passing the original differentiated nucleus through two rounds of early embryo development. In amphibians, recloning improved the developmental capacity of terminally differentiated nuclei, resulting in more advanced larval development compared to a single round of nuclear transfer [9]. However, in the experiments here, there was no improvement in terms of either embryo or fetal development compared to first-generation FBA cloned embryos derived directly from granulosa cells. The activation protocol in both the FBA and recloning groups was the same and involved a combination of ionomycin and 6-DMAP, although cytoplasts were activated at different ages: 30 and 24 hpm, respectively. Despite the difference in timing, the efficiency of oocyte activation in control oocytes, as evidenced by pronuclear formation, was the same (both 95%) and similar to that in a previous report [36]. In the experiments here, the blastomeres used for recloning were obtained from compacting morulae in excess of 30 cells. At this stage of development, the transcription of embryonic genes may have commenced in the first-generation cloned embryo [39]. It may be beneficial to reclone embryos before the expected onset of transcription. However, it is difficult at the 8-cell stage to visually identify those cloned embryos that are likely to have the potential for further development. Thus, compacting morulae were recloned, as our experience shows that good-quality morulae reliably develop into good-quality blastocysts. In the experiments here, preactivated (presumptive S-phase) cytoplasts, capable of accepting nuclei at any stage of the cell cycle [25], were fused with unsynchronized blastomeres. It may be necessary to investigate alternative cell cycle coordination options to improve developmental rates.

It is widely acknowledged that nuclear transfer, even with embryonic blastomeres, results in increased rates of abortion throughout pregnancy, high birth weight, perinatal deaths, and poor adaptation to extra-uterine life [40–42]. These effects appear more extreme with somatic cell nuclear transfer [1, 2, 10, 31, 32, 43] and may relate to one deficiency or a combination of deficiencies in either the nuclear transfer procedure itself, leading to incomplete nuclear reprogramming of the cultured donor cells, or in the in vitro maturation and embryo culture systems used. These deficiencies, either collectively or singularly, may lead to inappropriate patterns of gene expression at specific key stages during embryo, fetal, or placental development, contributing to pregnancy loss.

The survival of adult cloned embryos reconstructed in the FBA treatment 60 days after transfer, as indicated by ultrasonography, was relatively high (45%) in this study. This is similar to data from our laboratory with twin embryo transfer of bovine embryos produced after either nuclear transfer with quiescent fetal cells [12] or IVP [44], and is higher than with cloned embryos derived from either nonquiescent fetal cells [10] or embryonic blastomeres [33, 34]. However, the nuclear transfer process in cattle with both cultured embryonic [18] and fetal cells [12] and embryonic blastomeres [33, 34] is currently associated with

high rates of fetal loss throughout gestation. This was exemplified here, with 78% of adult cloned fetuses present at Day 60 not surviving to term, compared to a typical loss of 30% with bovine IVP embryos [23, 45]. It appears that the failure of normal placentation is a problem frequently observed with cloned embryos and also with a proportion of IVP embryos. Approximately 25% of the early embryonic mortality with the IVP embryo appears to be due to an unsuccessful transition from yolk sac to allantoic nutrition, whereby the growth of the allantois is severely retarded, or even nonexistent, and is characterized by a lack of vascularization by Day 34 [46]. It is therefore likely that the IVP cloned embryo will have a similar deficiency during this stage of development. There are reports of high fetal losses during the middle of the first trimester [12, 18]. Despite apparently normal fetal development, part of this loss may be due to a failure of normal placentome development [18]. These losses coincide with the stage at which functioning placentomes are required for the exchange of nutrients and gases [47], and this may in part be due to a deficiency in the underlying allantois (A.J. Peterson, personal communication). The majority of the fetal losses in the third trimester in this study were a consequence of hydrallantois in three twin-bearing recipient cows. This excessive accumulation of allantoic fluid may be a consequence of low numbers of placentomes, leading to placental dysfunction [48]. Reports in the literature with both IVP and cloned conceptuses describe increased incidence of hydrallantois in late gestation, fewer and enlarged placentomes, enlarged umbilical vessels, and edematous placental membranes [10, 42, 49, 50]. These abnormalities of placentation were all observed in some (but not all) recipients here. It is suggested that the embryo survival rate may have been greater had embryos in this study been transferred singularly to minimize pregnancy complications.

With the nuclear transfer pregnancies reported here, it appeared that the appropriate signaling in preparation for birth did not occur normally and there was a lack of "communication" between the maturing fetus and the recipient cow in the weeks leading up to expected full term (Day 282). This was characterized by few overt signs of readiness for birth and by inadequate mammary gland development in the recipient cows over this time. This may reflect another abnormality in placental function, as there are both direct and indirect actions of placental hormones on mammogenesis during pregnancy [51]. The deficiencies in parturition and mammogenesis noted here have been experienced by us previously with sheep [31, 32], and have prompted the current decision to deliver cloned offspring by elective cesarean section. Because of the decision to deliver calves between Days 276 and 281, and to not allow a sufficiently long period for them to calve following corticosteroid injection [52], we did not provide the opportunity to observe whether parturition would have been initiated after induction, or whether it might have occurred naturally at some later point in gestation. Offspring generated from both IVP [50, 53, 54] and cloned embryos [40, 42] do tend to have longer gestation lengths. If, however, there is a problem with either the fetal hypothalamic-anterior pituitary-adrenal axis and/or the transduction of the resulting rise in fetal cortisol near the time of birth to the cow [55], it certainly has not compromised the viability of the cloned calves themselves. Normally, the transmission of fetal cortisol to the dam is mediated by the changing activities of steroidogenic enzymes in the placenta [56], leading to an elevation in maternal estrogen, which in turn initiates the cascade of events leading to parturition [55]. Currently, it is not known whether the cloned calves had naturally high levels of cortisol, as the recipient cows each received an injection of corticosteroid 17 h before cesarean section in order to hasten fetal lung maturation [57]. Nevertheless, it is tempting to speculate that the lack of a typical parturition response may have been primarily due to the inability of the placenta to convert progesterone to estrogen.

The embryo culture system used here has no effect on the birth weight of IVP calves [23]. Despite the lack of control data, the birth weights observed here for the singles, but not the twins, tended to be greater than those generally reported in New Zealand for Friesians [58] and following IVP [23]. Although the birth weight of two cloned calves approached 50 kg, these were not as extreme as some reported elsewhere [40, 42]. All but one of the calves delivered were fundamentally viable and only required basic neonatal veterinary assistance. In the other case, the calf did require treatment to alleviate a slow, irregular heartbeat and respiration rate immediately after delivery. The immediate postnatal behavior of the cloned calves was more vigorous than previously reported [41], and all were standing within 30 min to 2 h and had suckled colostrum. It has been suggested that cloned calves exhibit defects in energy metabolism as evidenced by cases of hypothermia, hypoxemia, hypoglycemia, and metabolic acidosis, and that this may be a consequence of abnormal placental function [41]. With the calves reported here, oxygen therapy was provided by us as a standard procedure, and there was evidence of a metabolic imbalance immediately after birth in two of the calves, which was corrected without intervention.

While microsatellite DNA analyses are consistent with the cloned calves' being genetically identical to the Friesian donor cow (Fig. 2), there are differences in the black and white coat color patterns both between the calves and compared to the cow (Fig. 1, c and d). However, the variation is no different from that commonly observed in genetically identical twins that occur either naturally or after embryo bisection (Wells, personal observations). This aspect of piebald patterning appears not to be under absolute genetic control: environmental influences in utero result in a degree of variability in the multiplication and migration of melanoblasts, which form the melanocytes necessary for pigment production in the developing skin of the fetus [59, 60]. Subtle differences in the detail of the pigmentation markings between genetically identical cloned amphibians have also been noted [30].

The development of an efficient method for producing animals from cultured somatic cells after nuclear transfer will have a number of advantages for both agriculture and biomedicine [61, 62]. These particularly relate to new opportunities for introducing precise genetic modifications into livestock species, following homologous recombination in the cultured cells. We have identified a cell population in the adult female that has proven to be relatively successful in generating cloned offspring after nuclear transfer. Although mural granulosa cells do have a specialized function, they are not terminally differentiated, and, when combined with nuclear transfer methods whereby quiescent cells are fused before activation, relatively high rates of embryo development and survival have resulted. Overall, the technology is currently one-quarter the efficiency of IVP in the bovine species. To better enable the commercial application of this technology, however, the efficiency of the procedures needs to be substantially improved. Re-

search is needed to better understand the reprogramming of differentiated nuclei, in order to improve both the establishment and maintenance of healthy conceptuses. Studies should also focus on the development and function of the placenta and on parturition.

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Nuclear Transplantation in Early Pig Embryos[1]

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ABSTRACT

Nuclear transfer was evaluated in early porcine embryos. Pronuclear stage embryos were centrifuged, treated with cytoskeletal inhibitors, and subsequently enucleated. Pronuclei containing karyoplasts were placed in the perivitelline space of the enucleated zygote and fused to the enucleated zygote with electrofusion. The resulting pronuclear exchange embryos were either monitored for cleavage in vitro (9/13 cleaved and contained 2 nuclei after 24 h, 69%) or for in vivo development. In vivo development after 3 days resulted in 14/15 (93%) of the embryos transferred cleaving to the ≥4-cell stage and after 7 days 6/16 (38%) reaching the expanded blastocyst stage. A total of 56 pronuclear exchange embryos were allowed to go to term, and 7 piglets were born.

A similar manipulation procedure was used to transfer 2-, 4- or 8-cell nuclei to enucleated, activated meiotic metaphase II oocytes. Enucleation was effective in 74% (36/49) of the contemporary oocytes. Activation was successful in 81% (37/46) of nonmanipulated but pulsed oocytes versus 13% (4/31) of control oocytes ($p<0.01$). After 6 days in vivo, 9% (1/11) of the 2-cell nuclei, 8% (7/83) of the 4-cell nuclei, and 19% (11/57) of the 8-cell nuclei transferred to enucleated, activated meiotic metaphase II oocytes resulted in development to the compact morula or blastocyst stage ($p<0.01$). A total of 88 nuclear transfer embryos were transferred to recipient gilts for continued development. A single piglet was born after the transfer of a 4-cell nucleus to an enucleated, activated metaphase II oocyte and subsequent in vivo development. Therefore 4-cell nuclei are capable of directing development to term after transfer to an enucleated, activated meiotic metaphase II oocyte.

INTRODUCTION

The transfer of nuclei between the cells of early mammalian embryos has been accomplished in a variety of species, including mice (McGrath and Solter, 1983a; Robl et al., 1986), sheep (Willadsen, 1986), cattle (Prather et al., 1987; Robl et al., 1987), and rabbits (Stice and Robl, 1988), and has been described briefly for pig embryos (Robl and First, 1985). Nuclear transfer studies have served to describe nuclear versus cytoplasmic inheritance (McGrath and Solter, 1983b, 1984a; Mann, 1986; Robl et al., 1988) and imprinting during gametogenesis (Surani et al., 1986; Barra and Renard, 1988), as well as to determine the extent of nuclear differentiation in early development (Willadsen, 1986; Prather et al., 1987; Stice and Robl, 1988). Interestingly, the results obtained to date for all mammalian embryos other than mouse embryos suggest that nuclei from early cleavage stages can be reprogrammed to behave as 1-cell embryos if transferred to enucleated, activated meiotic metaphase II oocytes (reviewed by Prather and First, 1989).

Since all mammalian nuclei do not respond to nuclear transfer as mouse nuclei do, it is important to determine the affects of nuclear transfer in mammals other than the mouse. Here we report data showing that pronuclei can be exchanged successfully between porcine zygotes and result in normal offspring, and that some cleavage-stage nuclei are capable of directing complete development after transfer to an enucleated, activated meiotic metaphase II oocyte.

MATERIALS AND METHODS

Source of Embryos

Crossbred (Yorkshire × Landrace) gilts were monitored twice daily for signs of estrus. Oocytes were collected from nonmated animals 36 or 48 h after first-detected estrus, and pronuclear, 2-cell, 4-cell, and

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8-cell embryos were collected from mated animals at 48–72 h after the onset of estrus. Embryos were collected by flushing the oviducts and/or uteri with N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES)-buffered Tyrode's media (HbT; Bavister et al., 1983) supplemented with bovine serum albumin (3 mg/ml, Sigma, St. Louis, MO) and gentamicin sulfate (50 μg/ml; Sigma).

Macromanipulation

Micromanipulation of pronuclear embryos was completed as described for the bovine by Robl et al. (1987). Briefly, pronuclear stage embryos were centrifuged at 15,000 × *g* for 3 min to allow visualization of the pronuclei (Wall et al., 1985; Robl et al., 1987). Centrifuged pronuclear embryos were placed in HbT containing 7.5 μg/ml cytochalasin B (Sigma) and 0.1 μg/ml demecolcine (Sigma) for 5 min prior to macromanipulation. A single embryo was held in place by a holding pipette attached to a Narshigie micromanipulator. Next a 30–33 μm (external diameter, O.D.), beveled, sharpened, glass pipette, attached to another Narshigie micromanipulator, was inserted into the embryo and moved adjacent to the pronuclei. The pronuclei were then aspirated into the pipette, and the pipette was removed. This permitted the removal of the pronuclei within a membrane bound karyoplast. This karyoplast was transferred to an enucleated zygote. The membranes were then fused as described below.

Nuclear transfer procedures were carried out as described by Prather et al. (1987) in cows. Briefly, after embryos and oocytes were exposed to HbT containing cytochalasin B, but not demecolcine, oocytes were prepared by aspirating the first polar body and cytoplasm directly underneath (presumably containing the metaphase chromosomes) with a 30–33 μm (O.D.) pipette. A karyoplast from a 2-cell, 4-cell, or 8-cell stage embryo was then aspirated into the transfer pipette and expelled into the perivitelline space of the enucleated oocyte. Electrically induced membrane fusion was conducted as described below. Some oocytes were enucleated, stained, and evaluated for the presence of meiotic metaphase II chromosomes. Other oocytes were not manipulated but were exposed to similar conditions and then to the electrofusion pulse, or were sham-pulsed and cultured 24 h and examined for the presence of a single pronucleus.

Membrane Fusion

Membranes were fused by electrofusion (Prather et al., 1987; Robl et al., 1987). The nuclear transfer embryos were placed between two platinum electrodes 1 mm apart in a solution of 0.3 M mannitol (Sigma). The embryos were manually oriented so that the fusion plane was parallel to the electrodes, exposed to a 5 V AC (1000 KHz, 20% duty on) field for 5–10 s, and a 30-μs, 120 V/mm (DC) pulse was then applied. The AC field was programmed to decrease to 0 V over 5 s following the DC pulse. Power was provided by a Zimmermann Cell Fusion™ instrument (Model Z1000, GCA/Precision Scientific Group, Chicago, IL).

Culture

In vitro culture was conducted in 50-μl drops of TCM 199 supplemented with 10% heat-treated fetal bovine serum (Gibco, Grand Island, NY) under paraffin oil at 39°C under an atmosphere of 100% humidity and 5% CO_2 in air. Nuclear transfer embryos that had fused and were destined for in vivo culture were transferred to the oviducts of synchronized recipient gilts. In some gilts, pregnancy was terminated at slaughter and embryos were recovered by flushing the oviducts or uteri with HbT. These embryos were evaluated by phase-contrast microscopy or after fixation and staining with aceto-orcein and phase-contrast microscopy. Some of the blastocyst-stage embryos were transferred to the uteri of synchronized recipient gilts. In some cases, the recipient gilts were bred to a Hampshire boar to aid in maintaining the pregnancy by providing enough conceptuses while providing a color marking to identify offspring. Estrus was subsequently monitored twice daily with the aid of a boar.

Data were analyzed by chi-square (Snedecor and Cochran, 1980).

RESULTS

Pronuclear Exchange

Pronuclear exchanges were conducted to determine if the procedures developed for nuclear transfer were detrimental to continued development. Electrofusion was successful in 76% (89/117) of the manipulated zygotes. After 24 h of in vitro cultures, 9/13 (69%) of the pronuclear exchange embryos had cleaved and had 2 nuclei. After 3 days in vivo, 14/15 embryos contained 4 or more cells; these 15 embryos were subsequently transferred to another recipient gilt. Thirty-eight percent (6/16) of the pronuclear exchange embryos cultured in vivo for 7 days were recovered as expanded blastocysts:

four of these embryos were subsequently transferred to another recipient gilt. This resulted in a total of 56 pronuclear exchange embryos transferred to 6 recipient gilts that were allowed to continue to term (Table 1). One of these animals returned to estrus on Day 16, one on Day 50, and one on Day 93. The remaining 3 gilts farrowed a total of 32 pigs, 7 of which were derived from the transfer of 35 pronuclear exchange embryos.

Cloning

An examination of the steps involved with the methods for cloning revealed that 74% (36/49) of contemporary meiotic metaphase II oocytes were in fact enucleated. The pulse required for fusion activated 81% (37/46; as judged by the presence of a single pronucleus after 24 h of in vitro culture) of meiotic metaphase II oocytes that were not manipulated, whereas sham-pulsed oocytes activated 13% (4/31) of the time ($p<0.01$). Electrofusion rates were not significantly different between donor cell stages (2-cell, 59/77, 77%; 4-cell, 115/138, 83%; 8-cell, 71/83, 86%; $p>0.25$; this is a retrospective analysis).

In vitro development showed that 55% (11/20) of the 2- and 4-cell nuclei transferred, participated in a cleavage division (2-cell, 7/15; 4-cell, 4/5) after 24 h. After 6 days in vivo, 9% (1/11) of 2-cell donor nuclei, 8% (7/83) of 4-cell donor nuclei, and 19% (11/57) of the 8-cell donor nuclei ($p<0.01$) transferred to an enucleated, activated meiotic metaphase II oocyte had developed to the compact morula or expanded blastocyst stage (4 of the 4-cell donor nuclei-derived blastocyst-stage embryos were subsequently transferred to another recipient gilt; the other three were not transferred because a synchronized recipient was not available). A total of 42 nuclear transfer embryos were transferred to 4 nonbred recipients and allowed to continue pregnancy. One had a normal cycle, one had a 28-day cycle, and 2 had cycles of 52 days or more (Table 2). A total of 46 nuclear transfer embryos were transferred to 5 bred recipients. One 4-cell nuclear transfer piglet and forty-eight control piglets were born from these 5 gilts (Table 2).

TABLE 1. Establishment and maintenance of pregnancy after the transfer of pronuclear exchange embryos to recipient gilts.

Number transferred	Recipient	Pregnancy result
9	25[a]	estrus detected Day 16
8	1–4[a]	estrus detected Day 50
4[b]	17[c]	estrus detected Day 93
8	32[a]	4 pronuclear exchange pigs born 10 control pigs born
12	51[a]	2 pronuclear exchange pigs born 7 control pigs born
15[b]	7–7[a]	1 pronuclear exchange pig born 8 control pigs born

[a]Gilt was bred to a color-marked boar.

[b]Embryos were retransferred to a secondary recipient after collection from a primary recipient gilt.

[c]Gilt received 2 mg estradiol on Days 12 and 13 to maintain pregnancy (Pope et al., 1987).

TABLE 2. Pregnancy establishment and maintenance after the transfer of 2-, 4- or 8-cell nuclear transfer embryos to recipient gilts.

Number transferred	Donor cell stage	Recipient	Pregnancy result
10	2	25	extended cycle, 28 days
12	2	6809521	extended cycle, 52 days
11	2	509[a]	13 control piglets born
7[b]	4	55[a]	6 control piglets born 1 nuclear transfer pig born
4	4	234[c]	normal cycle
16	4	7352	extended cycle, 72 days
7	4	230[a]	11 control piglets born
17	8	46[a]	10 control piglets born
4	8	7372[a]	8 control piglets born

[a]Gilt was bred to a color-marked boar.

[b]Embryos were retransferred to a secondary recipient after collection from a primary recipient gilt.

[c]Gilt received 2 mg estradiol on Days 12 and 13 to maintain pregnancy (Pope et al., 1987).

DISCUSSION

The results presented in this paper show that pig zygotes can tolerate the conditions necessary for pronuclear exchange and continue development to term. The results further show that oocytes in meiotic metaphase II can be enucleated, activated, fused with a 4-cell karyoplast, and subsequently direct development to the blastocyst stage and to term.

Pronuclear exchange has been used to study the nuclear versus cytoplasmic inheritance of cell surface antigens (SSEA-3; McGrath and Solter, 1983b), lethal mutations (T^{hp}: McGrath and Solter, 1984a), imprinting (Surani et al., 1986), and control of early development (Robl et al., 1988). These studies were all conducted in mouse embryos, but the application of the techniques developed by McGrath and Solter (1983a) to the cow embryo (Robl et al., 1987) and now to the pig embryo permit similar investigations in these domestic species.

Nuclear transfer for cloning has been successful in embryos from amphibians (reviewed by Gurdon, 1986;

DiBerardino, 1987; Prather, 1989) as well as in embryos from sheep (Willadsen, 1986), cattle (Prather et al., 1987), rabbits (Stice and Robl, 1988), and mice (Tsunoda and Shioda, 1988), although it is not clear if any nuclear reprogramming has resulted from this nuclear transfer (McGrath and Solter, 1984b; Barnes et al., 1987; Howlett et al., 1987). A major developmental difference between these animal embryos is the timing of the transition from maternal control of development (relying upon maternally stored RNA) to zygotic control of development (relying upon zygotically produced RNA). The major transition appears to occur at the mid-blastula stage for *Xenopus* embryos (~4000-cell stage; Newport and Kirschner, 1982), 8- to 16-cell stage for sheep embryos (Calarco and McLaren, 1976; Crosby et al., 1988), 8- to 16-cell stage for cow embryos (Camous et al., 1986; Barnes, 1988; King et al., 1988) and 2-cell stage for mouse embryos (Bolton et al., 1984). Why nuclear transfer for cloning is successful in species other than the mouse is not known, but it is interesting to note that the transition to zygotic control of development for the mouse occurs at an earlier time than in the other species. Pig embryos appear to fall between cow, sheep, and mouse embryos and make a transition by the 8-cell stage (White et al., 1987; Prather et al., 1989b); they may begin producing rRNA by the 4-cell stage, since this is the time at which nucleoli are first seen to begin reticulating (Norberg, 1970).

In vivo development to the morula/blastocyst stage was lower for the 2-cell and 4-cell donor nuclei versus the 8-cell donor nuclei ($p<0.01$). However, because of the limited number of replications, high degree of variability between replications, and the fact that these are retrospective data, no firm conclusions should be inferred.

The fusion percentage was not significantly different for the different donor cell stages and resembles that found for the 2-cell to 8-cell stage bovine blastomeres (Prather et al., 1987).

A major complicating factor in conducting these experiments is the lack of tight control over the stage of the cell cycle at which the embryos are collected and manipulated. This should not present a problem for the pronuclear exchanges, because the zygotes should be close to the same stage of the cell cycle–probably all in S. However, for cloning, a single embryo collection may contain cells of 2 different cleavage stages, "late" 4-cell and "early" 8-cell embryos, e.g. 4-cell embryos and 5- to 8-cell embryos. In this paper, these late 4-cell donor nuclei were pooled with early 4-cell donor nuclei, e.g. a single collection containing 2-cell and 4-cell stage embryos. Although differences in stage of the cell cycle for nuclear transfer are not important for early amphibian nuclei (Ellinger, 1978), such effects of stage of the cell cycle are important for more slowly dividing cells (Von Beroldington, 1981), but this has not been evaluated in mammals. Future studies of ours will attempt to evaluate the differences between early and late cell stage donor nuclei.

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Developmental Biology

Production of calves by transfer of nuclei from cultured inner cell mass cells

(bovine embryonic stem cells/nuclear transfer/totipotency)

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ABSTRACT **We report here the isolation and *in vitro* culture of bovine inner cell mass (ICM) cells and the use of ICM cells in nuclear transfer to produce totipotent blastocysts that resulted in calves born. Of 15 cell lines represented in this study, 13 were derived from immunosurgically isolated ICM of 3 *in vitro* produced day 9–10 bovine blastocysts, while 2 lines were derived from single blastocysts. Approximately 70% of attempted cell lines became established cell lines when started from 3 ICMs. The ability to establish cell lines was dependent on the number of ICMs starting the line. Sire differences were noted in the ability of ICMs to establish cell lines and to form blastocysts. The cell lines were cultured as a low cell density suspension in the medium CR1aa plus selenium, insulin, and transferrin (SIT) and 5% fetal calf serum (FCS) for 6–101 days before use in nuclear transfer, at which time some had multiplied to more than 2000 cells. If allowed to aggregate, cells of established cell lines formed embryoid bodies. A total of 659 nuclear transfer clones were made by fusing the ES cells into enucleated oocytes with polyethylene glycol; 460 of these fused, based on cleavage (70%). After culture of the clones for 7 days *in vitro* in CR1aa/SIT/5% FCS, 109 (24%) of those fused became blastocysts. Thirty-four blastocysts were transferred into uteri of 27 cows, and 13 cows (49%) became pregnant. Four of the 13 cows gave birth to 4 normal calves. DNA typing showed the calves to be derived from the respective sires of the cell lines. The calves were derived from cultures of less than 28 days.**

The isolation and multiplication in culture of totipotent embryonic stem (ES) cells have value in providing a large population of identical cells for use by nuclear transfer to produce clonal offspring (1). ES cells also provide a mechanism for gene transfer by transfection, infection, or injection of genes into the cells (2–6). After insertion of a selectable marker, the transgenic cells can be separated and used either by chimerization into a blastocyst or through use as donor cells in nuclear transfer to produce transgenic offspring (5–7). In addition, homologous recombination techniques can be used with cultured ES cells to add or delete genes at specific sites in the genome (8–11).

All of the above have been accomplished only with ES cells of mice (6, 12). In mice, no offspring from presumed totipotent ES cells have been produced by conventional nuclear transfer (12, 13), although offspring were produced when mouse ES cells were chimerized with tetraploid mouse embryos (14).

For domestic animals, morphological identification of putative ES cells has been published (15–18). Pluripotency has been demonstrated for ES cells of swine (17–19), cattle (17, 20, 21), and sheep (19). Injection of newly isolated blastocyst inner cell mass (ICM) cells into other blastocysts has produced chimeric offspring in sheep (22) and cattle (23). Noncultured ICM cells appear to be totipotent as evidenced by blastocyst formation, pregnancies, and offspring after transfer into enucleated oocytes in rabbits (24), sheep (25), and cattle (26).

Cultured cells with ES cell characteristics have been transferred into bovine oocytes initially with the resulting 5-day cultured embryos surviving only to the 8-cell stage (27). More recently, bovine cell lines derived from ICM (20) or morulae (21) have produced pregnancies by nuclear transfer, which fail in the first trimester. Calves have been born from chimeric embryos but the ES cell contribution is as yet unknown. One chimeric fetus was ES cell positive (20). There are no published reports in domestic species that cultured ICM or putative ES cells are totipotent, as evidenced by offspring derived totally from these cells (6, 12, 17, 19–21, 27).

Most attempts to isolate and culture ICM cells have been based on or adapted from the original methods of Evans and coworkers for mice (2, 17). In general, these methods involve separation of blastocyst ICM from trophoblast trophectoderm cells by immunosurgery followed by isolation of cells with stem cell morphological characteristics from ICM cells as they plate down on a fibroblast feeder layer. The putative stem cells are then maintained as a colony on a monolayer of fibroblast cells with differentiation-inhibiting activity, leukemia inhibitory factor, buffalo rat liver (BRL) cells, or BRL conditioned medium added to inhibit differentiation. This system has allowed culture of pluripotent cells that can become embryoid bodies. Aggregated sheets of cells develop cellular beating heart activity. However, only in mice has it allowed demonstration of or maintenance of totipotency of the cultured cells [reviewed by Stewart (6) and Anderson (12)]. It has been suggested that these mouse-derived differentiation-inhibiting agents do not adequately prevent differentiation of stem cells in species other than rodents (12).

We report here the isolation and short-term *in vitro* culture of bovine ICM cells by using a different approach to prevent differentiation. These cells were used in nuclear transfer to produce blastocysts that resulted in the birth of normal calves. This result provides evidence of totipotency of cultured ICM cells in mammalian species other than mouse.

MATERIALS AND METHODS

All embryos used in this experiment were *in vitro* derived from slaughterhouse ovaries and frozen semen by the methods described by Sirard *et al.* (28), Parrish *et al.* (29), and Rosenkrans and First (30). Oocyte maturation was in TC199 containing 10% fetal calf serum (FCS) and 0.5 μg of NIH ovine luteinizing hormone (NIADDK-OLH-25) per ml. Oocytes were fertilized with sperm from any one of five different bulls

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Abbreviations: ES cells, embryonic stem cells; SIT, selenium, insulin, and transferrin; FCS, fetal calf serum; ICM, inner cell mass.

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with sperm concentration and heparin dose adjusted for each bull. At 40–48 hr postfertilization, embryos were manually stripped of all cumulus cells and extraneous sperm by repeated pipetting through a 190-μm fire-polished pipette. Subsequent embryo culture was carried out in a defined medium called CR1aa (30) for 7–8 days at 39°C in 5% CO_2/95% air with high humidity until the embryos had hatched or were fully expanded, after which they were subjected to immunosurgery. Embryos were first washed in 3 ml of TL Hepes with polyvinylpyrrolidone (Sigma; PVP-40) (1 mg/ml) and polyvinyl alcohol (Sigma; p8136) (1 mg/ml) and then washed through four or five CO_2 equilibrated microdrops (50 μl) of CR1aa, polyvinylpyrrolidone, and polyvinyl alcohol under paraffin oil.

Rabbit anti-bovine antibody (1:10 dilution; Sigma; B8270) was added at a 1:10 dilution for a final concentration of 1:100. Embryos were returned to the 39°C incubator for 30 min. The embryos were removed from the incubator and again washed through four or five fresh microdrops of medium. Then guinea pig complement (Sigma; S-1639) was added to the embryos at a 1:10 dilution from a 1:500 diluted stock for a final dilution of 1:5000. While in the presence of complement, the zonae pellucidae were removed by manual pipetting through a non-fire-polished 150-μm pipette tip. The remaining ICMs were washed and then one to three ICMs per 10-μl drop of the medium CR1aa plus SIT (sodium selenite, insulin, and transferrin; Sigma; I 1884) were placed under paraffin oil. Within 5 days, the ICMs started disassociating from a ball of cells into individual free-floating cells. At this time, the ball of cells was mechanically disaggregated by a micromanipulation needle. The medium was changed every 2–3 days by aspiration and replaced with fresh CO_2-equilibrated CR1aa with SIT. The addition of 5% FCS to the medium was beneficial in reducing the "stickiness" of these cells, allowing easier handling during micromanipulation. The ICM cells were maintained as disassociated cells in suspension culture for periods ranging from 1 week to 2 months, depending on the experimental protocol. The culture conditions were derived through a series of experiments comparing the effects of various growth factors, media, and medium supplements on cell maintenance and growth rates. Cell viability was determined by staining with propidium iodide. All embryos used to make cell lines were derived from embryos cultured in the CR1aa/SIT/5% FCS medium.

The cultured ICM cells were used as nuclear donor cells in nuclear transfer. Recipient oocytes were matured *in vitro* (29) and stripped of cumulus 16–18 hr after initiation of oocyte maturation, using hyaluronidase at a concentration of 2 mg/ml and a fire-polished pipette. Oocytes were selected for the presence of polar bodies and returned to maturation medium for another 2–4 hr. Nuclear transfer was begun ≈20 hr after these metaphase II oocytes were placed into culture. Manipulation was performed with a Nikon Diaphot microscope equipped with Hoffman optics and Narishige micromanipulators. Manipulation was done in culture dishes in which microdrops of medium were arranged with each dish containing both 100-μl drops (TL Hepes with Ca^{2+} and Mg^{2+}) in which the oocytes were placed and 20-μl drops (TL Hepes with Ca^{2+} and Mg^{2+} and 20–50% FCS) to one side containing the cultured ICM cells. This was done to prevent the cells from sticking to the oocytes and to prevent mistaking ICM cells with any remaining cumulus cells. Approximately 10 ICM cells were aspirated into the transfer pipette, and then the tips were moved to the drop containing the oocytes. The cells were drawn higher into the pipette to allow space for enucleation of the oocyte. The oocyte was positioned on a holding pipette so that the polar body was toward the transfer tip. A small amount of cytoplasm from the region directly beneath the polar body and the polar body were removed. The transfer tip was retracted from the zona and the cytoplasm was ejected. The tip was reinserted through the same hole and an ICM cell was deposited beneath the zona. The cell was pressed against the plasma membrane, where it stuck firmly between the zona and plasma membrane. Due to the extreme stickiness of the cells, transfer pipettes were changed frequently. Nuclear (ICM cell) transfer was completed by 24 hr postmaturation, and the unfused units were placed in CR1aa medium overnight. All fusions were done with oocytes 42 hr postfollicular removal.

Fusion proved to be a difficult problem because of the disparate sizes of the cells to be fused. The ICM cells ranged in size from 15 to 25 μm, and the enucleated oocytes were ≈140 μm. Except for Table 4, in which recent experiments (bulls F–I) used electrofusion, fusions were with polyethylene glycol (PEG). The fusion protocol used PEG (M_r 1300–1600; Sigma) 1:0.25 g/ml in Ca^{2+}- and Mg^{2+}-free TL Hepes with polyvinyl alcohol (1 mg/ml) for 45 sec followed by a 1:1 dilution in the same medium for 1 min, another 1:1 dilution for 2 min, and then a final 1:1 dilution for 2–3 min. The most reliable PEG was from Boehringer Mannheim (PEG 1500). A 15-min culture in TL Hepes containing 20% FCS allowed membranes to return to their normal appearance. To activate the ooplasm, the embryos were washed through Ca^{2+}- and Mg^{2+}-free TL Hepes and then exposed to 5 mM ionomycin (Calbiochem) in 1 ml of medium for 45 sec. This was followed by another 15-min culture in TL Hepes containing 20% FCS, after which embryos were returned to CR1aa medium for further maturation.

RESULTS

To prevent differentiation, ICM cells were cultured in suspension at a concentration sufficiently low (1000–1500 cells per 10-μl drop) so that cell aggregation and differentiation did not occur. Several differentiation-inhibiting and mitotic factors were tested in various media combinations for their ability to promote prolonged mitotic activity of ICM cells cultured in loose suspension. Only media consisting of CR1aa plus SIT and either glucose, rifampicin, laminin, or 5% FCS supported mitosis through 2 weeks of culture. Of these, only CR1aa plus SIT plus 5% FCS allowed mitosis and continued proliferation of ICM cells for 4 weeks. ICM cells from day 9 and 10 bovine blastocysts multiplied in culture when cultured in CR1aa plus SIT and 5% FCS with some lines reaching 2000 cells after 2 weeks of culture. These cells have the appearance of mouse ES cells, being small cells with large nuclei, little cytoplasm, and prominent nucleoli (Fig. 1). When

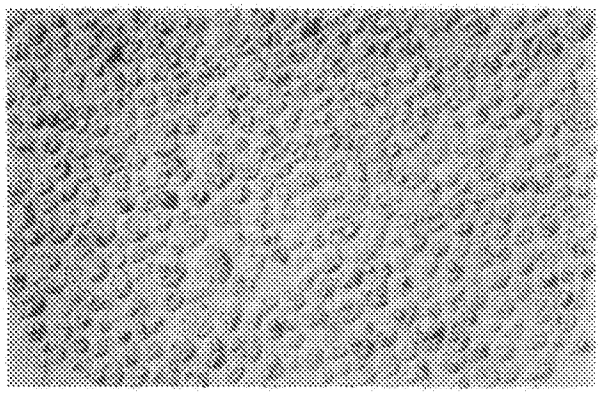

FIG. 1. Bovine ES cells. Cell population in 10-μl microdrops varied from 200 to 2000 cells within 2 weeks of culture. Individual lines were subcultured at 1000–1500 cells per microdrop because embryoid bodies formed when cell population densities exceeded 1000 cells. Note that the nucleus constitutes most of the volume of each cell, the presence of two or three nucleoli per cell, and the large round cells that will soon divide.

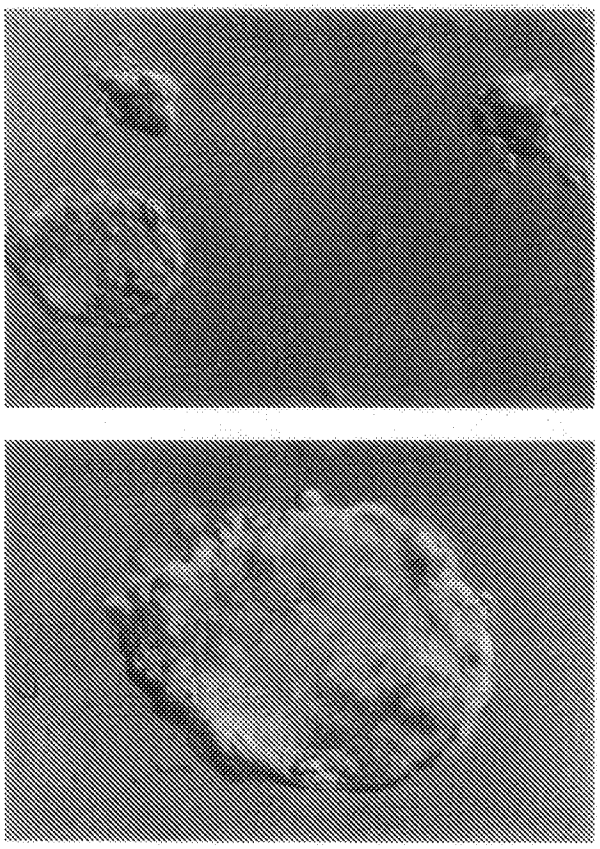

FIG. 2. Embryoid bodies resulting from high cell population density. (*Upper*) Bovine embryoid bodies, two simple and one complex. (*Lower*) Bovine complex embryoid body.

removed from nondifferentiating conditions and allowed to aggregate, the cultured cells formed embryoid bodies (Fig. 2).

Table 1. Effect of number of ICM cells starting a culture on ICM cell survival

No. of ICMs starting cell line	No. of cell lines	
	Started	Surviving at 1 month
1	159	0
3	241	170 (70.54%)

These embryoid bodies do not appear to differ morphologically from mouse embryoid bodies.

The ability of ICM cells cultured in loose suspension microdrops to establish and maintain a proliferating population of ICM cells appears to be dependent on the number of ICMs used to establish the culture (Table 1). ICM cultures derived from three ICMs from two sires established cultures that proliferated, whereas none of the cultures started from one ICM survived to 10 days and even the surviving cultures had a low proliferation rate, with most dying by 1 month. Occasionally, a single ICM initiated a cell culture as the two shown in Table 2. While one cell line, line six, was maintained for 101 days, most ICM lines derived from pooled embryos lost life and nuclear staining after 3 weeks of culture. Live/dead staining at 3 weeks with calcein AM (live) or ethidium homodimer (dead) showed ≈80% live cells (green) and 20% dead (red) and dying (orange-yellow) cells, whereas, at 5 weeks, nearly 80% of the cells were dead or dying.

One way of accurately determining totipotency of embryonic cells is to fuse the cell in question into an enucleated metaphase II oocyte. We report in Table 2 results from derivation and use of cells from 15 bovine ICM cell lines in nuclear transfer. The cell lines ranged from 6 to 101 days of culture at the time of nuclear transfer. A total of 659 embryos (clones) were made by nuclear transfer. After culture for 7 days *in vitro* in CR1aa and SIT plus 5% FCS, 109 became blastocysts (16.6%); of those cleaving, 25% became blastocysts. Each cell line was derived from the ICM of 3 blastocysts except cell lines 14 and 15, which were each derived from the ICM of a single blastocyst. The efficiency of establishing stem cell cultures from ICM cells and the ability of the oocyte ICM cell fusion product to become a blastocyst

Table 2. Use of loose suspension cultured ICM cells as donors of nuclei in nuclear transfer to produce blastocyts

Cell line	Days PIS to nuclear transfer*	Nuclear transfer clones made†	Cleavage (%)	Blastocysts, n‡	Blastocysts, % of clones§	Blastocysts, % of cleavage
1	35	24	14/24 (58)	6	25	43
2	42	32	20/32 (63)	4	12.5	20
3		33	21/33 (64)	4	12	19
4	17	92	71/92 (77)	19	21	27
5	71	22	18/22 (82)	4	18	22
6	101	22	15/22 (61)	4	18	27
7	6	44	36/44 (82)	9	20	25
7	6	33	2/33 (6)	0	0	0
8	13	57	43/57 (75)	11	19	26
9	20	42	20/42 (48)	6	14	30
10	27	74	61/74 (82)	12	16	20
11	14	23	17/23 (74)	4	17	24
11	14	5	4/5 (80)	2	40	50
12	21	47	40/47 (85)	4	8.5	10
13		28	21/28 (75)	6	21	29
14¶	54	39	21/39 (54)	6	15	29
15¶	61	42	36/42 (86)	8	19	22
	Total	659	460/659 (70)	109	15%	24%

*PIS, postimmunosurgery. Trophoblast cells were removed and culture of ICM cells was initiated.
†Each clone is the product of attempted fusion of an ICM cell with an enucleated oocyte.
‡Number of blastocysts after *in vitro* culture of the clones for 7 days.
§Frequency of clones becoming blastocysts after 7 days of culture.
¶All cell lines were derived from the pooled ICM of 3 blastocysts, except lines 14 and 15, each of which was derived from the ICM of a single blastocyst.

Table 3. Effect of sire on efficiency of stem cell line production and frequency of blastocysts derived from fusion of stem cells into enucleated oocytes [nuclear transfer (NT)]

	Sire breed	NT clones made, *n*	NT clones becoming blastocysts	
			n	%
A	Angus	89*	14	15.7
B	Holstein	114*	23	20.2
C	Holstein	272*	42	15.4
D	Brahman	184*	30	16.3
E	Brahman	46†	0	0
F	Longhorn	93†	14	15
G	Holstein	102†	22	22
H	Holstein	60†	6	10
I	Holstein	88†	19	22

*Stem cells were fused into enucleated oocytes by using PEG.
†Stem cells were fused into enucleated oocytes by electrofusion.

appears to be partially dependent on the genetics of the embryo as indicated by differences among sires in the frequency of stem cell line formation and blastocyst formation (Table 3).

Totipotency of cultured ICM cells from five cultured cell lines was determined by transfer into cows of blastocysts derived from ICM cell nuclear transfer (Table 4).

Thirty-four of 42 blastocysts derived from cell lines cultured for 6, 13, 20, 27, or 101 days were transferred into uteri of 27 cows. Thirteen of the cows (49%) became pregnant. At 180 days of gestation, 5 (19%) were still carrying 5 (15%) fetuses with heart beats clearly imaged with ultrasonography. Four of the cows delivered normal calves derived from the cultured ICM cells after gestations of normal length. The birth weights of the calves were 75, 80, and 85 pounds for 3 female calves and 86 pounds for a male calf. The gestations were 279, 280, 280, and 279 days, respectively.

The cell cultures producing offspring were cultures 7, 9, and 10. Cell culture 7 was derived from embryos sired by Holstein bull 9805 and the calf born from this culture was Holstein. Cell culture 9 was derived from embryos sired by Brahman bull 9813 and the two calves born were half Brahman. Cell culture 10 was derived from embryos sired by Longhorn bull 12199 and the calf was half Longhorn. DNA typing by Marijo Kent (31) established that each calf was sired by the sire producing the ICM cells from which the calf was derived. The calves were karyotyped and two half-sister Brangus calves from cell line 9 showed tetraploidy of <10% in some lymphocytes at birth but lost the tetraploid lineage by 1 year of age. Karyotypes of integument fibroblasts were normal.

DISCUSSION

The ICM cell culture system reported here prevents differentiation by culturing cells as a loose suspension with <1500 cells per 10-μl drop. Without cell–cell contact, neither cell aggregation nor monolayer formation occurred.

The results presented in Table 3 show that at least some of the ICM cells retain totipotency after culture. The efficiency of blastocyst production from use of the cultured ICM cells in nuclear transfer (15% or 25% of cleaved) is similar to the efficiency of using morulae cells as the donated nucleus in conventional nuclear transfer (18%; ref. 32).

The frequencies of pregnancies (49%) and 180-day maintained pregnancies (19%) after transfer into cows of embryos derived from cultured ICM cells were also similar to the frequency of pregnancies (30%) or maintained pregnancies reported for conventional bovine nuclear transfer (32, 33). The frequency of transferred blastocysts resulting in born offspring was also similar to bovine nuclear transfer and the calves were from three different cell lines.

Pluripotency has been demonstrated previously for cultured cattle ICM cells (12, 18, 20, 21, 27). Our present research was reported as an abstract in 1993 (34). This work demonstrates totipotency from cultured ICM cells of domestic animals, as evidenced by offspring, and the successful use of cultured cells for nuclear transfer (for review of other species, see ref. 12). The methods presented here allowed establishment of ICM cell lines from ≈70% of the blastocysts attempted, when the line was formed from a pool of 3 ICMs. This is approximately equal to the best efficiencies in the production of mouse ES cell lines (6). In mice, some ES cell lines have been shown to be of abnormal karyotype, particularly after several passages. Whether the tetraploidy of calves from line 9 was due to nuclear transfer or ICM cell culture is unknown. While the efficiency of fusion was acceptable in this study (68%), manufacturers' lots of PEG vary considerably in fusogenic activity. A modified electrofusion procedure was used with success for production of blastocysts from cells derived from sires E, F, G, H, and I in Table 3.

The greatest need will be for cell culture systems that promote much greater mitotic activity than the present system while inhibiting differentiation. The CR1aa/SIT/5% FCS culture medium used for this study is adequate only for short-term culture and represents a mere beginning in identification of an optimal culture system for bovine ICM cells.

With the development of culture systems allowing a high rate of cell multiplication, bovine ES cells derived from ICMs or earlier embryo stages should prove useful in propagation and genetic modification of cattle. The use of ES cells in gene transfer could provide more efficient gene transfer with opportunities to select cells for gene integration or expression before offspring are made and opportunities through homol-

Table 4. Production of calves from blastocysts derived from fusion of cultured ICM cells into enucleated bovine oocytes

Cell line*	Days PIS to NT†	NT clones made, *n*	Blastocysts from NT,‡ *n*	Blastocysts (*n*) transferred into cows (*n*)	Cows pregnant at 42 days gestation, *n*	Blastocysts surviving as fetuses *in utero* at gestation day				Calves born
						56	70	150	180	
7	6	44	9	9 into 6	4	5	4	2	2	1
8	13	57	11	6 into 4	0	—	—	—	—	—
9	20	42	6	6 into 4	3	4	4	4	2	2
10	27	74	12	9 into 9	3	1	1	1	1	1
6	101	22	4	4 into 4	3	0	—	—	—	—
			Total	34 into 27	13 of 27 (49%)	10	9 (27%)	7 (21%)	5 (15%)	4 (12%)

*Each of these cell lines was established from the pooled ICMs of 3 blastocysts.
†PIS, postimmunosurgery and start of ICM cell cultures.
‡Number of blastocysts developed per number of clones made after 7 days of culture.

ogous DNA recombination to be site specific in gene transfer or deletion. Bovine ES cells when used as nuclear donors in nuclear transfer could allow the production of large numbers of clonal offspring from one valuable embryo or from the genetically modified ES cells of one valuable embryo.

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In vitro Embryo Culture in the Production of Identical Merino Lambs by Nuclear Transplantation

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Abstract

This study examined the viability of embryos developed *in vitro* from 8- to 16-cell stage blastomeres fused with enucleated oocytes. Of 209 blastomeres recovered and subjected to manipulation and electrofusion procedures, 190 (91%) fused successfully, with 86 (45%) of those undergoing cleavage up to the 4- to 16-cell stage when cultured for 66 h in a synthetic oviduct fluid medium. The viability of the embryos was examined by transferring them to recipient ewes and determining the ewes' pregnancy status by ultrasound on Day 45. Of 86 embryos transferred, 14 developed to fetuses in 8 of the 36 recipients, including four sets of identical twins and one set of quads. In contrast, with uncultured and unmanipulated embryos, 15 fetuses developed from 19 embryos transferred at a similar stage of development. The viability of embryos derived from manipulated zygotes cultured *in vitro* was comparable to that previously reported for studies employing *in vivo* culture, indicating the potential of *in vitro* culture systems based on a simple medium for nuclear-transplantation embryos.

Introduction

Production of identical sheep is possible through the transplantation of cleavage stage nuclei into enucleated oocytes (Willadsen 1986; Smith and Wilmut 1989). To minimize losses of cloned embryos due to damage to the zona pellucida from the manipulation procedures, zygotes have been temporarily transferred to ovine oviducts encased in agar gel capsules and recovered at the morula–blastocyst cell stage for transfer to recipient ewes (Willadsen 1986). Recently, several procedures have been developed that allow *in vitro* culture of ovine zygotes for up to 3 days, one such procedure being the use of a simple medium based on the composition of sheep oviduct fluid (Walker *et al.* 1988). This paper examines the use of *in vitro* culture as a means of both simplifying procedures for handling zygotes created by nuclear transplantation prior to transfer and allowing detailed study of the early developmental stages.

Materials and Methods

Oocyte and Embryo Collection

Superovulation was induced in New South Wales Merino ewes with 12-day progestagen pessaries (60 mg medroxyprogesterone acetate each; Upjohn Pty Ltd, Sydney, New South Wales) and 22 mg per ewe of follicle-stimulating hormone (Heriot Agvet Pty Ltd, Melbourne, Victoria). Synthetic gonadotrophin-releasing hormone (50 mg i.m. per ewe; Intervet Australia Pty Ltd, Artarmon, New South Wales) was administered 30 h after pessary removal. Ewes were inseminated via a laparoscope (Walker *et al.* 1984) with a minimum of 20×10^6 motile fresh spermatozoa per uterine horn 48 h after pessary removal.

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Oocytes and embryos were collected by mid-ventral laparotomy approximately 12–17 h and 60 h, respectively, after the expected median time of ovulation. Oviducts were flushed with 10 mL Dulbecco's phosphate-buffered saline (Flow Laboratories, North Ryde, New South Wales) containing 10% heat-inactivated sheep serum. Oocytes and embryos were recovered from the flushings within 5 min of collection.

Culture and Transport

Embryos were cultured in synthetic oviduct fluid medium (SOFM) according to the formulation of Tervit *et al.* (1972), but with 20% heat-inactivated human serum (HS) in lieu of bovine serum albumin (Walker *et al.* 1986). Transportation of embryos was in tubes containing SOFM–HS supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid). The transport medium was pre-equilibrated in an atmosphere of 5% CO_2 in air at 37°C, and transport between the collection site and the laboratory was accomplished at 37°C. The time between collection and initiation of manipulation was 2–4 h. Embryos were cultured after manipulation in 10 μL drops of SOFM–HS under paraffin oil in 35 mm plastic dishes in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. The microdrop culture was pre-equilibrated in the culture environment 24 h prior to the addition of embryos. Incubation was maintained at 38·5°C and 95% relative humidity.

Manipulation Procedures

Manipulations were carried out at room temperature (25°C). The manipulation medium for donor-nuclei embryos and oocytes was SOFM supplemented with 25 mM HEPES, 5 mM $NaHCO_3$, 7·5 mg mL^{-1} cytochalasin B (CB; Sigma, St Louis, Missouri), and 10% heat-inactivated sheep serum. Donor-nuclei embryos and oocytes were placed in SOFM–CB for 15 min prior to manipulation. Isolation of donor-nuclei blastomeres, enucleation of oocytes, and blastomere transfer were essentially as described by Prather *et al.* (1987).

Electrofusion was performed with an apparatus similar to that described by Kubiak and Tarkowski (1986) but modified by the addition of an a.c. pulse generator (Topward TFG-8104, Taiwan). Electrical output was monitored with an oscilloscope (Goodwill GOS-522B, Sydney, New South Wales). For fusion, manipulated embryos were placed in a solution of 0·3 M mannitol, 0·1 M $MgSO_4$, and 0·05 mM $CaCl_2$ (Willadsen 1986) for 10 min and positioned between platinum electrodes placed 200 mm apart. Manipulated embryos were then aligned in an a.c. field of 3 V, 500 kHz, followed by a 13 V d.c. pulse of 100 ms duration, followed by a reduction of the a.c. field strength to 0 V over 15 s after the d.c. pulse. The embryos were then cultured in SOFM–CB for 1 h to increase development (Smith and Wilmut 1989), and fusion rates were recorded before the embryos were placed in the SOFM–HS microdrop culture. Embryos were observed for cleavage every 24 h after fusion. To determine the number of viable embryos remaining after selection on the basis of morphological criteria (see below), the remaining embryos from the last three trials were cultured a further 56 h and the number of nuclei determined by staining with Hoechst 33342 (Sigma, St Louis, Missouri) (Pursel *et al.* 1985) and examination with fluorescence microscopy (200×).

Embryo Transfers

Manipulated embryos were selected from culture at 66 post-fusion on the basis of morphological appearance and cleavage regularity during culture. One to five embryos were transferred to individual ewes according to recipient availability, condition of embryos, and number of clones derived from each donor embryo. Embryos collected at the same stage as donor embryos were also transferred to synchronized recipients within 2–3 h of collection to test the survival of uncultured and unmanipulated embryos. Recipient animals were tested for pregnancy by ultrasound on Day 45 to determine the number of fetuses *in utero*.

Results

A total of 31 donor embryos from seven ewes were selected for nuclear transfer, yielding 209 successfully manipulated blastomeres. Of these, 190 (91%) fused, and 86 (45%) of those underwent cleavage up to the 4- to 16-cell stage during 66 h of culture and were subsequently transferred to 36 recipient ewes. Nuclei counts in embryos not selected for transfer (84) indicated that 40 (48%) had undergone cleavage to various stages (Table 1).

with 12 (14%) of those forming blastocysts following a further 54 h of culture. The cell numbers of blastocysts forming in culture ranged from 6 to 32.

At Day 45, 8 (22%) of the 36 recipients receiving the manipulated and cultured embryos were pregnant (Table 2), and 14 fetuses (16% of the 86 embryos transferred) were detected by ultrasound. Transfer of untreated control embryos (19) to recipients (7) resulted in all animals becoming pregnant with a total of 15 fetuses (79%) *in utero*. Amongst the pregnancies from manipulated and cultured embryos, there were six sets of twins and two singletons. Each set of twins was derived from a single embryo, with two of the sets originating from the same embryo.

Table 1. *In vitro* development of untransferred embryos at 120 h post-fusion

Embryos cultured	2–16 cells	>16 cells	Blastocysts	Nuclei per blastocyst[A]
84	33 (39%)	7 (8%)	12 (14%)	14 ± 7·1 (6–32)

[A] Mean ± s.d.; range in parentheses.

Table 2. Development of manipulated embryos cultured for 66 h post-fusion and of control embryos of similar age

	Embryos transferred[A]	Pregnancies	Fetuses
Manipulated	86 (36)	8 (22%)	14 (16%)[B]
Control	19 (7)	7 (100%)	15 (79%)

[A] Number of recipients in parentheses.
[B] Significantly different from control group (χ^2 analysis; $P < 0{\cdot}01$).

Discussion

The results indicate that the *in vitro* culture system developed by Walker *et al.* (1988) can be used to support the early development of nuclear-transplantation embryos. The pregnancy rate achieved (22%) is similar to that obtained with *in vivo* culture of nuclear-transfer embryos from sheep (17%; Smith and Wilmut 1989) and cattle (22·5%; Bondioli *et al.* 1990), with the major loss of embryos occurring early in pregnancy at either implantation or shortly thereafter. Unmanipulated ovine embryos cultured in the same system for a similar period *in vitro* and transferred produce high numbers of elongated conceptuses (94%) at Day 14 (Walker *et al.* 1988). Thus, the losses experienced with micromanipulated embryos more likely relate to the manipulation procedures than to deficiencies in the culture system or to the inability of the donor nuclei to be reprogrammed sufficiently to support development.

Overall embryo survival after transfer was 16%, with 70% of embryos forming fetuses in recipients that became pregnant and with a higher number of twin than singleton pregnancies (6:2). In mouse embryos, variation in the cell cycle stage of donor nuclei can influence development following transfer to the enucleated zygote (Smith *et al.* 1988), and this may account for the large variation seen in the rate of development of manipulated sheep embryos in culture. This variation may cause losses due to asynchrony between embryos and recipients (Rowson *et al.* 1966).

The morphological criteria used to screen embryos after 66 h of *in vitro* culture identified the majority of the embryos as suitable for transfer, with only 7 (8%) of the 84 remaining

embryos undergoing further, albeit impaired, development as assessed by the low cell numbers. There are no comparable reports on the survival of ovine embryos cultured *in vitro* following nuclear transfer, but bovine nuclear-transfer embryos have been maintained to the blastocyst stage in SOFM–HS (Seamark, unpublished observations) and in co-culture (Bondioli *et al.* 1990), with subsequent pregnancies. Further extension of the culture period for the manipulated embryos prior to transfer was not attempted as experience with unmanipulated embryos (Gandolfi and Moor 1987; Walker *et al.* 1988) indicates that this would probably result in a further reduction in the pregnancy rate.

The present study indicates that the simple *in vitro* culture system described may provide a viable alternative to the *in vivo* culture systems that have previously been employed for short-term culture of manipulated zygotes. If, as the present data suggest, the *in vitro* system is as efficacious as the *in vivo* systems, it can be recommended as the method of choice since it is not only simpler to manage but also has the advantage of permitting direct observation of embryos, thus enabling experimentation aimed at improving understanding of the factors that limit the early critical stages of embryo development. Further studies aimed at providing a direct comparison of the efficacies of the *in vitro* and *in vivo* systems are warranted.

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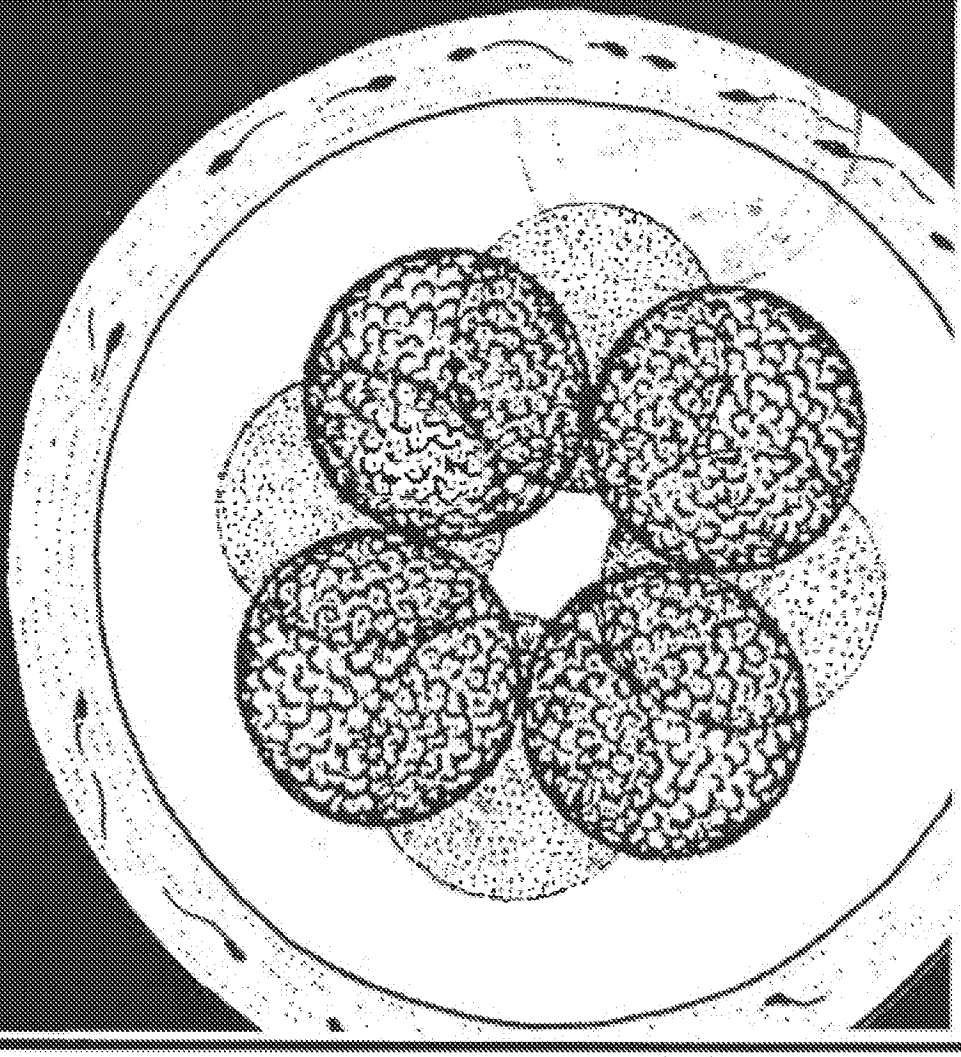

NUCLEAR TRANSPLANTATION IN GOATS

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It has been reported that offspring of sheep, cattle, rabbit and pig have been produced by nuclear transplantation. But a similar report has never been published for the goat. The purpose of the present study was to examine the developmental capacity of nuclear transplanted embryos produced by transfer of blastomeres from 4 to 32-cell embryos into enucleated oocytes matured in vivo.

Matured oocytes and 4-32-cell embryos were recovered surgically from superovulated Shaanbei black goats and Guanzhong Saanen dairy goats, respectively. Nuclear transplantation procedures were performed as described by Willadsen in for sheep (Nature 310:63). First the polar body, metaphase chromsomes and about half of the cytoplasm were removed from the oocyte by aspiration with a beveled glass pipette. Donor embryos were mechanically separated into individual cells by micromanipulation. Each blastomere was transferred into the zona pellucida (ZP) of the enucleated oocyte with the same beveled glass pipette. The micromanipulation was performed in phosphate buffered saline (PBS) containing 5 μg/ml cytochalasin B. The karyoplast oocyte was placed in PBS in a fusion chamber and fusion was induced with a 45 V DC pulse of 40 μsec duration. Fused nuclear transplanted embryos were inserted into another ZP and transferred after culture for 1 to 3 h in vitro in PBS with 15% fetal calf serum at 37°C.

Results of karyoplast oocyte fusion and nuclear transplanted embryo transfer

Donor embryo stage	No. attempted fusion	No. successful fusion	No. transferred embryos	No. off-spring
4-cell	7	5	5	1
8-cell	8	6	6	1
16-cell	8	7	7	1
32-cell	7	6	6	2 a)
total	30	24	24	5

a) identical twins

The present study showed that nuclear transplanted goat embryos produced by tranfer of a blastomere from 4 to 32-cell embryos into an enucleated matured oocyte can develop into normal kids in vivo; the commercial cloning of goats will be a reality in the near future.

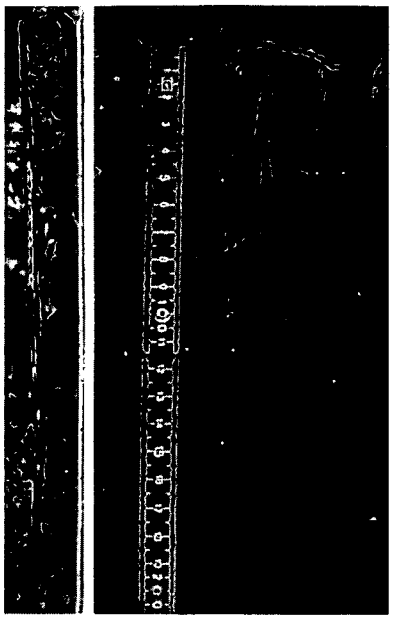

Figure 5 Spear II, which is 2.30 m long. The spear is shown to the left of an incomplete pelvis of a horse, and the base has been broken off. Inset shows a detail of the tip of spear II. Scale in cm.

isotope stage 7, and are at least of stage 9. Correlations of the Schöningen sequence to other areas[12,14] suggest that they were deposited during the fourth-last interglacial, probably at the end of stage 11. Judging from the mammalian fauna, the Reinsdorf assemblage is younger than the Lower Palaeolithic site of Boxgrove (West Sussex, UK)[19,20].

Before our discovery, the oldest complete 'spear' known was discovered in Eemian deposits at Lehringen (Lower Saxony, Germany) in 1948 (ref. 2). Thought to be about 125 kyr old (oxygen-isotope substage 5e), this thrusting spear was recovered from between the ribs of a straight-tusked elephant, and was made from yew (*Taxus*). The Schöningen spears are probably three full interglacial/glacial cycles older than the Lehringen lance (Fig. 2).

The discovery of spears designed for throwing means that theories of the development of hunting capacities and subsistence strategies of Middle Pleistocene hominids must be revised, as well-balanced, sophisticated hunting weapons were common from an early period of the Middle Pleistocene onwards. Accordingly, meat from hunting may have provided a larger dietary contribution than has previously been acknowledged[21,22]. The Schöningen evidence also illustrates how little is known about the 'organic' component of early hominid material culture. □

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Viable offspring derived from fetal and adult mammalian cells

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Fertilization of mammalian eggs is followed by successive cell divisions and progressive differentiation, first into the early embryo and subsequently into all of the cell types that make up the adult animal. Transfer of a single nucleus at a specific stage of development, to an enucleated unfertilized egg, provided an opportunity to investigate whether cellular differentiation to that stage involved irreversible genetic modification. The first offspring to develop from a differentiated cell were born after nuclear transfer from an embryo-derived cell line that had been induced to become quiescent[1]. Using the same procedure, we now report the birth of live lambs from three new cell populations established from adult mammary gland, fetus and embryo. The fact that a lamb was derived from an adult cell confirms that differentiation of that cell did not involve the irreversible modification of genetic material required for development to term. The birth of lambs from differentiated fetal and adult cells also reinforces previous speculation[1,2] that by inducing donor cells to become quiescent it will be possible to obtain normal development from a wide variety of differentiated cells.

It has long been known that in amphibians, nuclei transferred from adult keratinocytes established in culture support development to the juvenile, tadpole stage[3]. Although this involves differentiation into complex tissues and organs, no development to the adult stage was reported, leaving open the question of whether a differentiated adult nucleus can be fully reprogrammed. Previously we reported the birth of live lambs after nuclear transfer from cultured embryonic cells that had been induced into quiescence. We suggested that inducing the donor cell to exit the growth phase

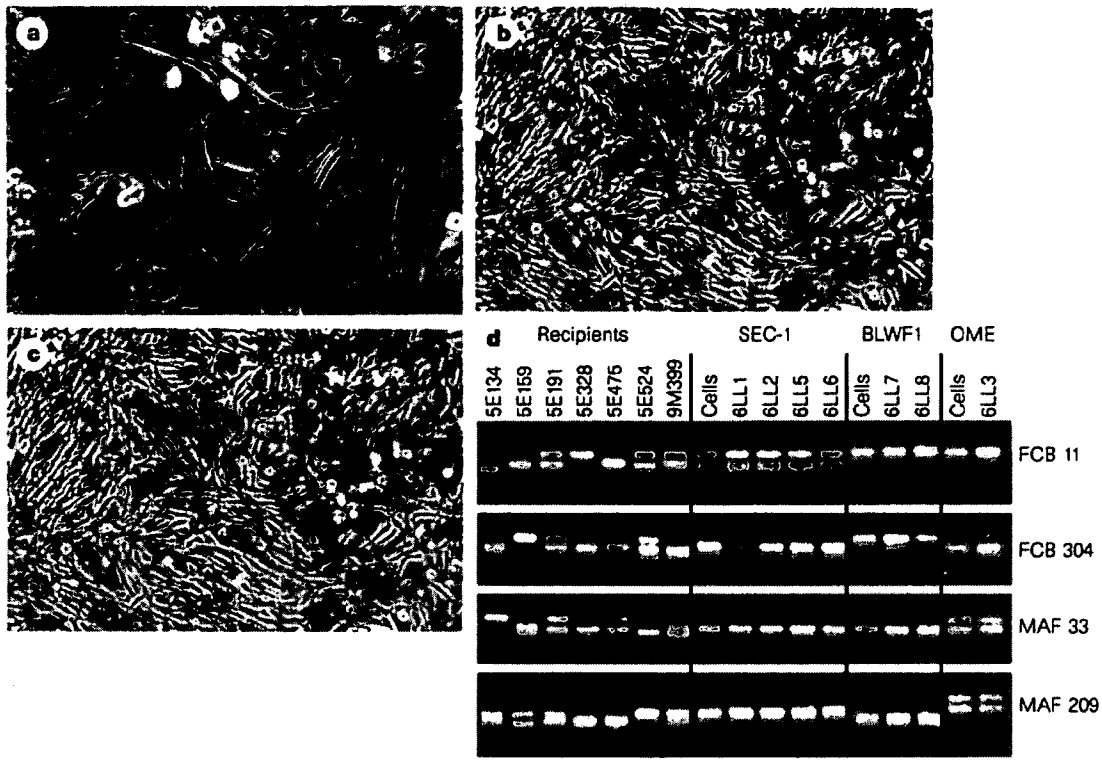

Figure 1 Phase-contrast photomicrograph of donor-cell populations: **a**, Embryo-derived cells (SEC1); **b**, fetal fibroblasts (BLWF1); **c**, mammary-derived cells (OME). **d**, Microsatellite analysis of recipient ewes, nuclear donor cells and lambs using four polymorphic ovine markers[22]. The ewes are arranged from left to right in the same order as the lambs. Cell populations are embryo-derived (SEC1), fetal-derived (BLW1), and mammary-derived (OME), respectively. Lambs have the same genotype as the donor cells and differ from their recipient mothers.

causes changes in chromatin structure that facilitate reprogramming of gene expression and that development would be normal if nuclei are used from a variety of differentiated donor cells in similar regimes. Here we investigate whether normal development to term is possible when donor cells derived from fetal or adult tissue are induced to exit the growth cycle and enter the G0 phase of the cell cycle before nuclear transfer.

Three new populations of cells were derived from (1) a day-9 embryo, (2) a day-26 fetus and (3) mammary gland of a 6-year-old ewe in the last trimester of pregnancy. Morphology of the embryo-derived cells (Fig. 1) is unlike both mouse embryonic stem (ES) cells and the embryo-derived cells used in our previous study. Nuclear transfer was carried out according to one of our established protocols[1] and reconstructed embryos transferred into recipient ewes. Ultrasound scanning detected 21 single fetuses on day 50–60 after oestrus (Table 1). On subsequent scanning at ~14-day intervals, fewer fetuses were observed, suggesting either mis-diagnosis or fetal loss. In total, 62% of fetuses were lost, a significantly greater proportion than the estimate of 6% after natural mating[4]. Increased prenatal loss has been reported after embryo manipulation or culture of unreconstructed embryos[5]. At about day 110 of pregnancy, four fetuses were dead, all from embryo-derived cells, and post-mortem analysis was possible after killing the ewes. Two fetuses had abnormal liver development, but no other abnormalities were detected and there was no evidence of infection.

Eight ewes gave birth to live lambs (Table 1, Fig. 2). All three cell populations were represented. One weak lamb, derived from the fetal fibroblasts, weighed 3.1 kg and died within a few minutes of birth, although post-mortem analysis failed to find any abnormality or infection. At 12.5%, perinatal loss was not dissimilar to that occurring in a large study of commercial sheep, when 8% of lambs died within 24 h of birth[6]. In all cases the lambs displayed the morphological characteristics of the breed used to derive the nucleus donors and not that of the oocyte donor (Table 2). This

Table 1 Development of embryos reconstructed with three different cell types

Cell type	No. of fused couplets (%)*	No. recovered from oviduct (%)	No. cultured	No. of morula/ blastocyst (%)	No. of morula or blastocysts transferred†	No. of pregnancies/ no. of recipients (%)	No. of live lambs (%)‡
Mammary epithelium	277 (63.8)[a]	247 (89.2)	-	29 (11.7)[a]	29	1/13 (7.7)	1 (3.4%)
Fetal fibroblast	172 (84.7)[b]	124 (86.7)	-	34 (27.4)[b]	34	4/10 (40.0)	2 (5.9%)
			24	13 (54.2)[b]	6	1/6 (16.6)	1 (16.6%)§
Embryo-derived	385 (82.8)[b]	231 (85.3)	-	90 (39.0)[b]	72	14/27 (51.8)	4 (5.6%)
			92	36 (39.0)[b]	15	1/5 (20.0)	0

* As assessed 1 h after fusion by examination on a dissecting microscope. Superscripts a or b within a column indicate a significant difference between donor cell types in the efficiency of fusion ($P < 0.001$) or the proportion of embryos that developed to morula or blastocyst ($P < 0.001$).
† It was not practicable to transfer all morulae/blastocysts.
‡ As a proportion of morulae or blastocysts transferred. Not all recipients were perfectly synchronized.
§ This lamb died within a few minutes of birth.

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Figure 2 Lamb number 6LL3 derived from the mammary gland of a Finn Dorset ewe with the Scottish Blackface ewe which was the recipient.

Table 2 Delivery of lambs developing from embryos derived by nuclear transfer from three different donor cells types, showing gestation length and birth weight

Cell type	Breed of lamb	Lamb identity	Duration of pregnancy (days)*	Birth weight (kg)
Mammary epithelium	Finn Dorset	6LL3	148	6.6
Fetal fibroblast	Black Welsh	6LL7	152	5.6
	Black Welsh	6LL8	149	2.8
	Black Welsh	6LL9†	156	3.1
Embryo-derived	Poll Dorset	6LL1	149	6.5
	Poll Dorset	6LL2‡	152	6.2
	Poll Dorset	6LL5	148	4.2
	Poll Dorset	6LL6‡	152	5.3

* Breed averages are 143, 147 and 145 days, respectively for the three genotypes Finn Dorset, Black Welsh Mountain and Poll Dorset.
† This lamb died within a few minutes of birth.
‡These lambs were delivered by caesarian section. Overall the nature of the assistance provided by the veterinary surgeon was similar to that expected in a commercial flock.

alone indicates that the lambs could not have been born after inadvertent mating of either the oocyte donor or recipient ewes. In addition, DNA microsatellite analysis of the cell populations and the lambs at four polymorphic loci confirmed that each lamb was derived from the cell population used as nuclear donor (Fig. 1). Duration of gestation is determined by fetal genotype[7], and in all cases gestation was longer than the breed mean (Table 2). By contrast, birth weight is influenced by both maternal and fetal genotype[8]. The birth weight of all lambs was within the range for single lambs born to Blackface ewes on our farm (up to 6.6 kg) and in most cases was within the range for the breed of the nuclear donor. There are no strict control observations for birth weight after embryo transfer between breeds, but the range in weight of lambs born to their own breed on our farms is 1.2–5.0 kg, 2–4.9 kg and 3–9 kg for the Finn Dorset, Welsh Mountain and Poll Dorset genotypes, respectively. The attainment of sexual maturity in the lambs is being monitored.

Development of embryos produced by nuclear transfer depends upon the maintenance of normal ploidy and creating the conditions for developmental regulation of gene expression. These responses are both influenced by the cell-cycle stage of donor and recipient cells and the interaction between them (reviewed in ref. 9). A comparison of development of mouse and cattle embryos produced by nuclear transfer to oocytes[10,11] or enucleated zygotes[12,13] suggests that a greater proportion develop if the recipient is an oocyte. This may be because factors that bring about reprogramming of gene expression in a transferred nucleus are required for early development and are taken up by the pronuclei during development of the zygote.

If the recipient cytoplasm is prepared by enucleation of an oocyte at metaphase II, it is only possible to avoid chromosomal damage and maintain normal ploidy by transfer of diploid nuclei[14,15], but further experiments are required to define the optimum cell-cycle stage. Our studies with cultured cells suggest that there is an advantage if cells are quiescent (ref. 1, and this work). In earlier studies, donor cells were embryonic blastomeres that had not been induced into quiescence. Comparisons of the phases of the growth cycle showed that development was greater if donor cells were in mitosis[16] or in the G1 (ref. 10) phase of the cycle, rather than in S or G2 phases. Increased development using donor cells in G0, G1 or mitosis may reflect greater access for reprogramming factors present in the oocyte cycoplasm, but a direct comparison of these phases in the same cell population is required for a clearer understanding of the underlying mechanisms.

Together these results indicate that nuclei from a wide range of cell types should prove to be totipotent after enhancing opportunities for reprogramming by using appropriate combinations of these cell-cycle stages. In turn, the dissemination of the genetic improvement obtained within elite selection herds will be enhanced by limited replication of animals with proven performance by nuclear transfer from cells derived from adult animals. In addition, gene targeting in livestock should now be feasible by nuclear transfer from modified cell populations and will offer new opportunities in biotechnology. The techniques described also offer an opportunity to study the possible persistence and impact of epigenetic changes, such as imprinting and telomere shortening, which are known to occur in somatic cells during development and senescence, respectively.

The lamb born after nuclear transfer from a mammary gland cell is, to our knowledge, the first mammal to develop from a cell derived from an adult tissue. The phenotype of the donor cell is unknown. The primary culture contains mainly mammary epithelial (over 90%) as well as other differentiated cell types, including myoepithelial cells and fibroblasts. We cannot exclude the possibility that there is a small proportion of relatively undifferentiated stem cells able to support regeneration of the mammary gland during pregnancy. Birth of the lamb shows that during the development of that mammary cell there was no irreversible modification of genetic information required for development to term. This is consistent with the generally accepted view that mammalian differentiation is almost all achieved by systematic, sequential changes in gene expression brought about by interactions between the nucleus and the changing cytoplasmic environment[17]. □

Methods

Embryo-derived cells were obtained from embryonic disc of a day-9 embryo from a Poll Dorset ewe cultured as described[1], with the following modifications. Stem-cell medium was supplemented with bovine DIA/LIF. After 8 days, the explanted disc was disaggregated by enzymatic digestion and cells replated onto fresh feeders. After a further 7 days, a single colony of large flattened cells was isolated and grown further in the absence of feeder cells. At passage 8, the modal chromosome number was 54. These cells were used as nuclear donors at passages 7–9. Fetal-derived cells were obtained from an eviscerated Black Welsh Mountain fetus recovered at autopsy on day 26 of pregnancy. The head was removed before tissues were cut into small pieces and the cells dispersed by exposure to trypsin. Culture was in BHK 21 (Glasgow MEM; Gibco Life Sciences) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM) and 10% fetal calf serum. At 90% confluency, the cells were passaged with a 1 : 2

division. At passage 4, these fibroblast-like cells (Fig. 1) had modal chromosome number of 54. Fetal cells were used as nuclear donors at passages 4–6. Cells from mammary gland were obtained from a 6-year-old Finn Dorset ewe in the last trimester of pregnancy[18]. At passages 3 and 6, the modal chromosome number was 54 and these cells were used as nuclear donors at passage numbers 3–6.

Nuclear transfer was done according to a previous protocol[1]. Oocytes were recovered from Scottish Blackface ewes between 28 and 33 h after injection of gonadotropin-releasing hormone (GnRH), and enucleated as soon as possible. They were recovered in calcium- and magnesium-free PBS containing 1% FCS and transferred to calcium-free M2 medium[19] containing 10% FCS at 37 °C. Quiescent, diploid donor cells were produced by reducing the concentration of serum in the medium from 10 to 0.5% for 5 days, causing the cells to exit the growth cycle and arrest in G0. Confirmation that cells had left the cycle was obtained by staining with antiPCNA/cyclin antibody (Immuno Concepts), revealed by a second antibody conjugated with rhodamine (Dakopatts).

Fusion of the donor cell to the enucleated oocyte and activation of the oocyte were induced by the same electrical pulses, between 34 and 36 h after GnRH injection to donor ewes. The majority of reconstructed embryos were cultured in ligated oviducts of sheep as before, but some embryos produced by transfer from embryo-derived cells or fetal fibroblasts were cultured in a chemically defined medium[20]. Most embryos that developed to morula or blastocyst after 6 days of culture were transferred to recipients and allowed to develop to term (Table 1). One, two or three embryos were transferred to each ewe depending upon the availability of embryos. The effect of cell type upon fusion and development to morula or blastocyst was analysed using the marginal model of Breslow and Clayton[21]. No comparison was possible of development to term as it was not practicable to transfer all embryos developing to a suitable stage for transfer. When too many embryos were available, those having better morphology were selected.

Ultrasound scan was used for pregnancy diagnosis at around day 60 after oestrus and to monitor fetal development thereafter at 2-week intervals. Pregnant recipient ewes were monitored for nutritional status, body condition and signs of EAE, Q fever, border disease, louping ill and toxoplasmosis. As lambing approached, they were under constant observation and a veterinary surgeon called at the onset of parturition. Microsatellite analysis was carried out on DNA from the lambs and recipient ewes using four polymorphic ovine markers[22].

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Evidence against a dedicated system for word learning in children

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Children can learn aspects of the meaning of a new word on the basis of only a few incidental exposures and can retain this knowledge for a long period—a process dubbed 'fast mapping'[1–8]. It is often maintained that fast mapping is the result of a dedicated language mechanism, but it is possible that this same capacity might apply in domains other than language learning. Here we present two experiments in which three- and four-year-old children and adults were taught a novel name and a novel fact about an object, and were tested on their retention immediately, after a 1-week delay or after a 1-month delay. Our findings show that fast mapping is not limited to word learning, suggesting that the capacity to learn and retain new words is the result of learning and memory abilities that are not specific to language.

In two experiments (study 1 and study 2), 48 three-year-old children (mean age, 3 yr 7 months), 47 four-year-old children (mean age, 4 yr 5 months) and 48 undergraduate students first participated in a training phase that lasted for about twenty minutes. This phase involved the manipulation of ten kinds of objects, four of them familiar (for example, pennies) and six of them novel (see Methods). Subjects were asked to use some of the objects to measure other objects: for instance, they were asked to use pennies to measure the circumference of a plastic disc. Children were told it was a game, and adults were told it was a game designed to teach young children how to measure.

In the course of the training phase, subjects in both study 1 and study 2 were exposed to a new word—'koba'—used to refer to one of the six unfamiliar kinds of objects. Subjects in study 1 were also taught a new fact about one or more objects belonging to another kind. They were told that the object or objects was given to the experimenter by her uncle. Subjects in study 2 were given information about an unfamiliar object, presented visually. They watched as a sticker was placed on one of the unfamiliar objects, and were told that was where the sticker should go (see Methods).

In each of the studies, one-third of the subjects from each age group were tested for comprehension immediately after the training phase, one-third were tested after a 1-week delay (6–8 days), and one-third after a 1-month delay (28–30 days). Subjects were presented with the original array of ten items and asked to recall which object was the koba. Subjects in study 1 were also asked to recall which object was given to the experimenter by her uncle. Subjects in study 2 were handed a small sticker and instructed to put it where it should go (see Methods).

United States Patent [19]

Flom et al.

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[45] **Date of Patent: Feb. 3, 1987**

[54] **IRIS RECOGNITION SYSTEM**

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[21] Appl. No.: **703,312**

[22] Filed: **Feb. 20, 1985**

[51] **Int. Cl.**[4] **G06K 9/00**

[52] **U.S. Cl.** **382/2**; 351/205; 351/206; 354/62; 382/6

[58] **Field of Search** 382/2, 6; 351/206, 221, 351/208, 205; 354/62; 362/227, 231

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Primary Examiner—Leo H. Boudreau
Assistant Examiner—Joseph Mancuso
Attorney, Agent, or Firm—Ostrolenk, Faber, Gerb & Soffen

[57] **ABSTRACT**

Methods and apparatus are disclosed for identifying an eye, especially a human eye, on the basis of the visible features of the iris and pupil. The eye is first illuminated until the pupil reaches a predetermined size, at which an image of the iris and pupil is obtained. This image is then compared with stored image information for identification. The stored image information is previously obtained from an eye, the pupil of which was similarly brought to the same predetermined size. The illumination of the iris may include oblique illumination from several positions around the circumference of the iris. The illumination from each position may be relatively monochromatic, so that the resulting shadow will lack the color of the light source at that position, providing better contrast for elevation-depednent features. A system for performing iris recognition may include a processor which controls an illumination control circuit and a camera to obtain images at several predetermined sizes of the pupil.

32 Claims, 12 Drawing Figures

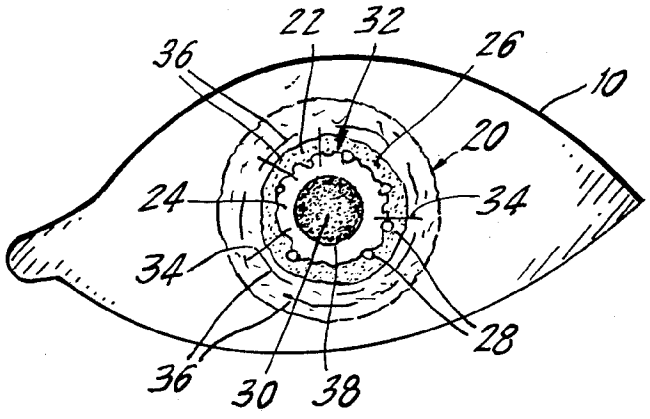


FIG. 1.

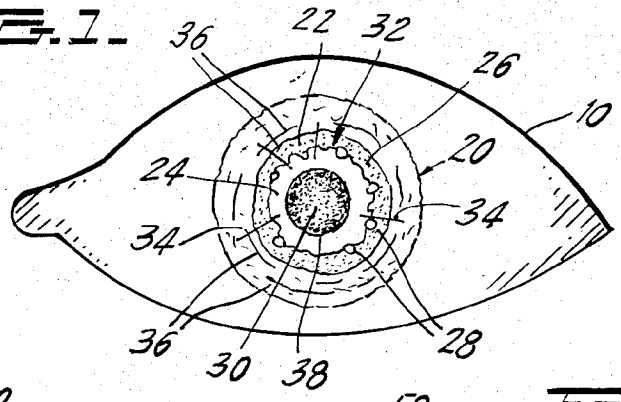


FIG. 2.

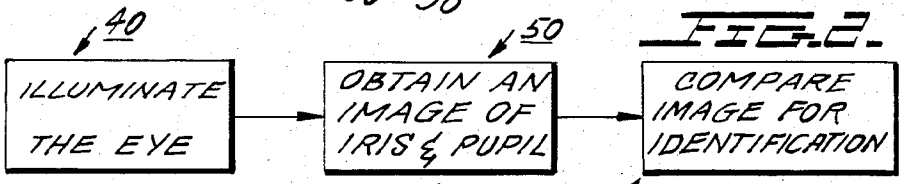


FIG. 3.

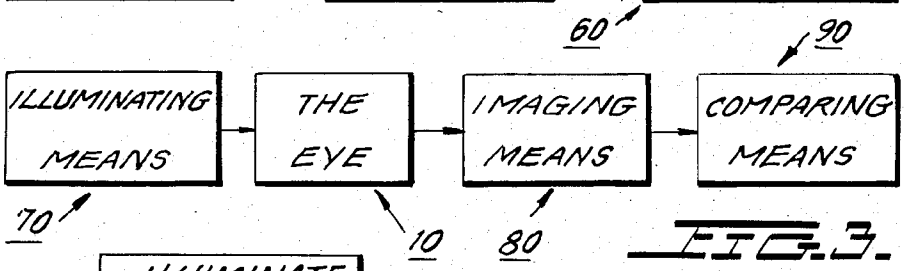


FIG. 4.

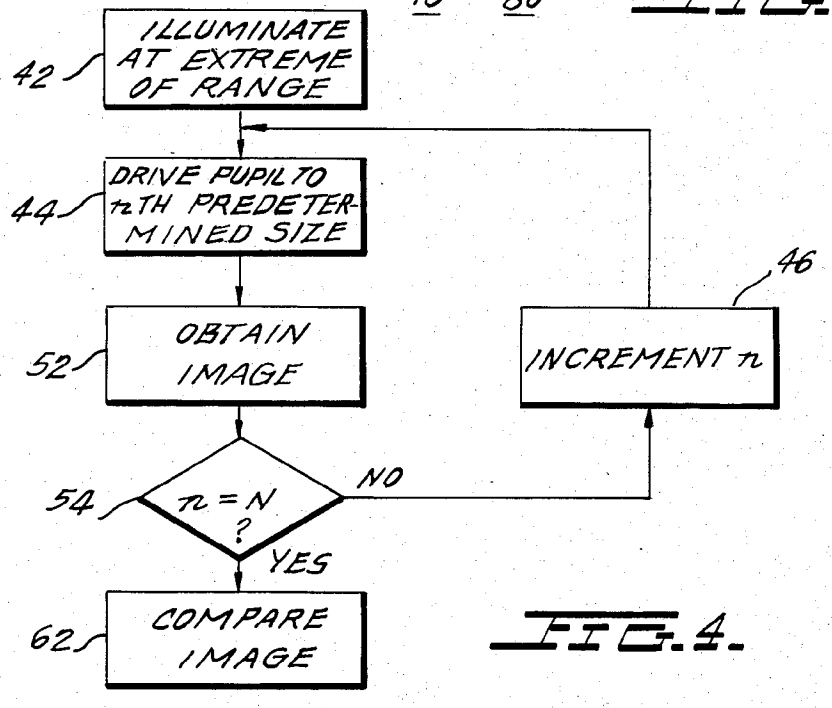


FIG. 5

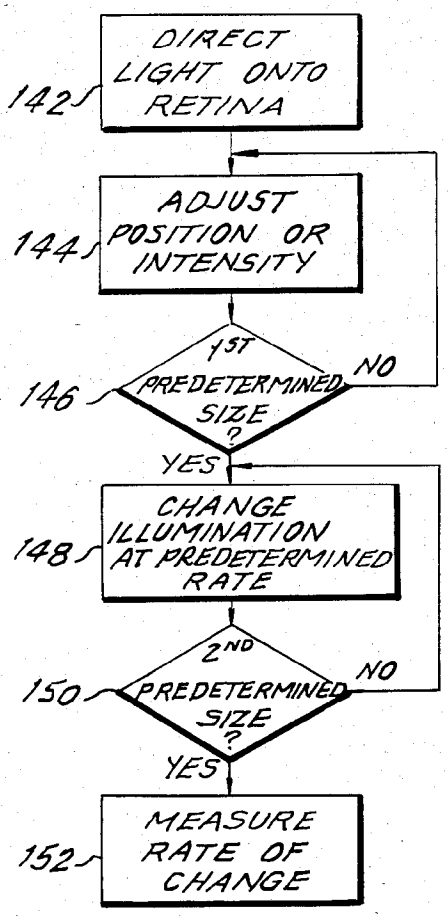


FIG. 7

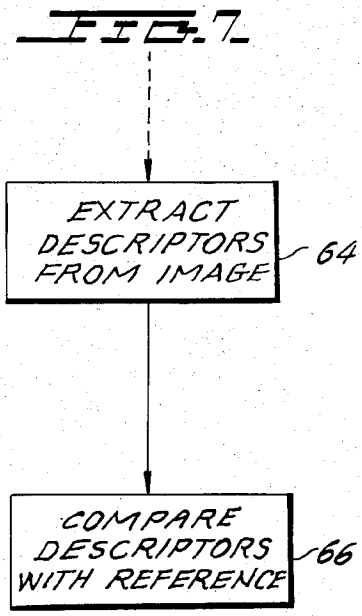


FIG. 6

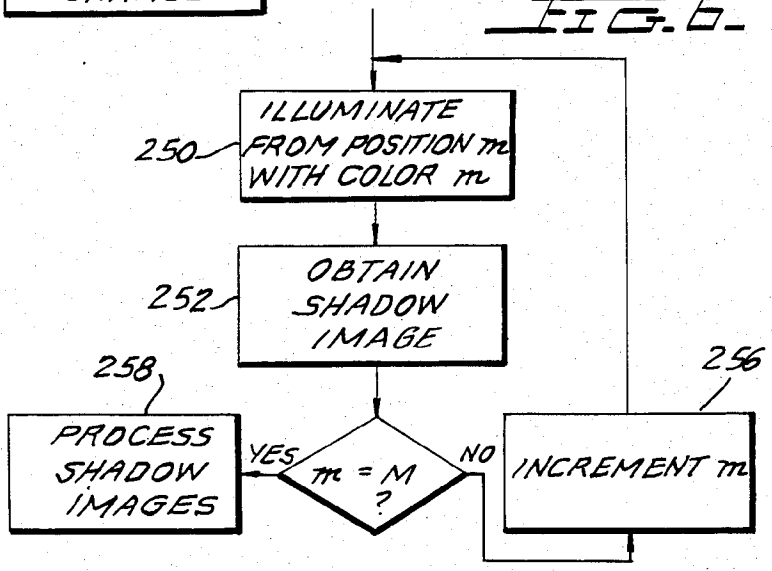


FIG. 8.

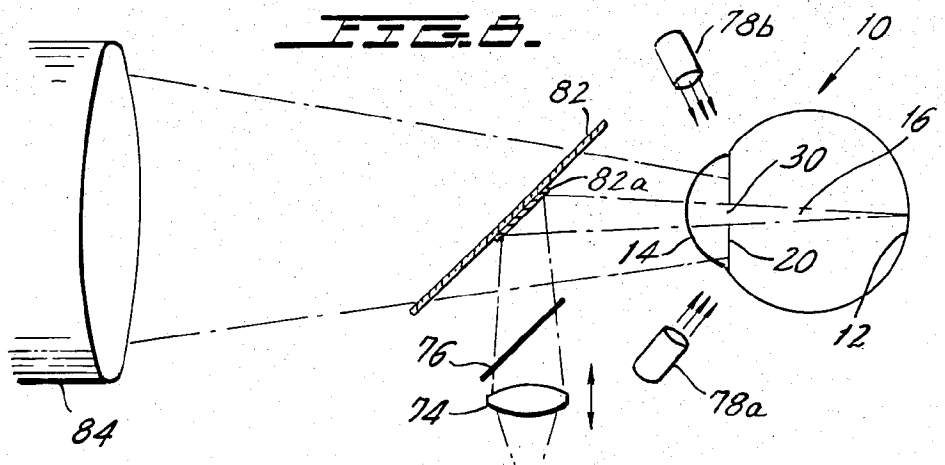


FIG. 9.

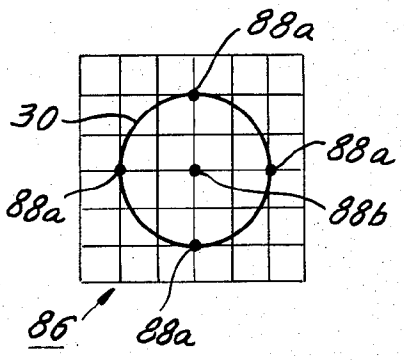


FIG. 10.

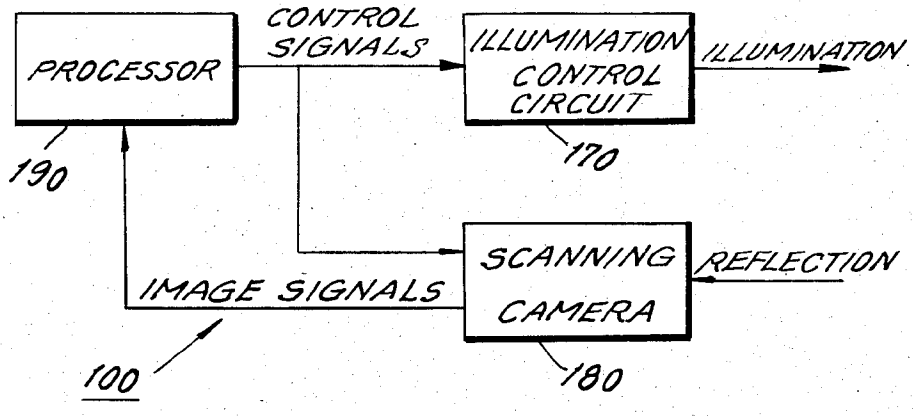


FIG. 12

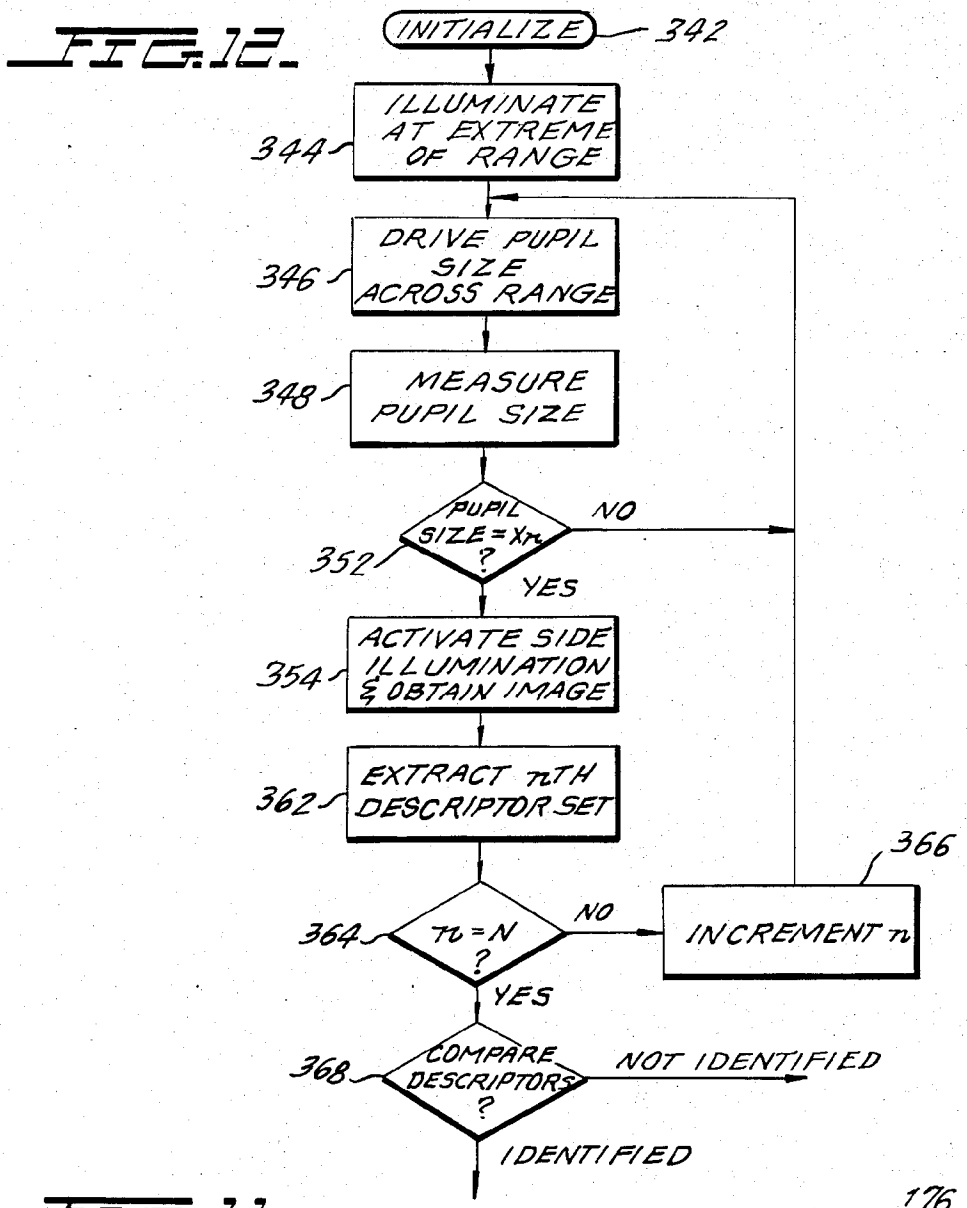
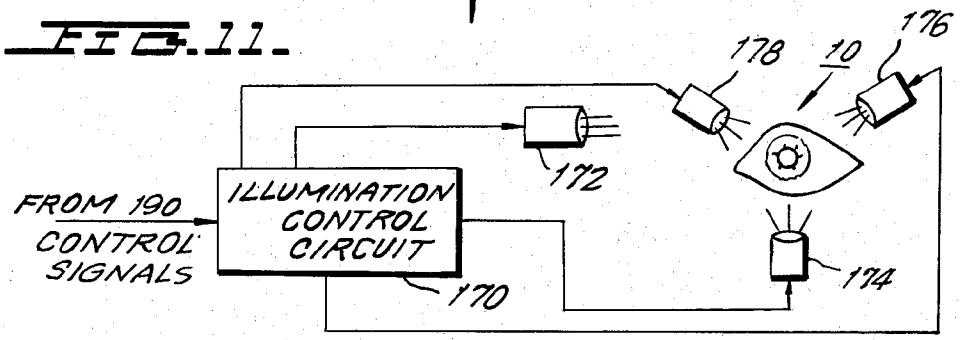

INITIALIZE
342
ILLUMINATE AT EXTREME OF RANGE
344
DRIVE PUPIL SIZE ACROSS RANGE
346
MEASURE PUPIL SIZE
348
PUPIL SIZE = Xn ?
352
NO
YES
ACTIVATE SIDE ILLUMINATION & OBTAIN IMAGE
354
EXTRACT nTH DESCRIPTOR SET
362
366
n = N ?
364
NO
INCREMENT n
YES
COMPARE DESCRIPTORS ?
368
NOT IDENTIFIED
IDENTIFIED

FIG. 11


178
10
176
172
FROM 190
CONTROL SIGNALS
ILLUMINATION CONTROL CIRCUIT
170
174

IRIS RECOGNITION SYSTEM

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to methods and apparatus for identification of physical characteristics of a human being or other animal. More specifically, the invention relates to the recognition of the physical characteristics of the eyes.

2. Description of the Prior Art

Several known techniques are traditionally used to identify a human being from physical characteristics. Fingerprints are one of the best known because of their extensive use by law enforcement agencies. Identification by fingerprint is especially helpful where an individual has left his fingerprint during the commission of a crime, but in general, the technology of fingerprint identification relies heavily on human judgment. In addition, the collection and detection of fingerprints is typically difficult.

More recently, U.S. Pat. No. 4,109,237 has disclosed an identification technique making use of the retinal vasculature patterns of the human eye. Among the advantages of this technique are that it can be automated, and is simpler than detecting, collecting, and identifying fingerprints. Furthermore, although some individuals may attempt to alter their fingerprints in order to avoid identification, altering the retinal vasculature pattern would be difficult and dangerous, and might impair or destroy the vision in that eye.

It would be advantageous to have an identification technique making use of the unique characteristics of the eye but not requiring pupil dilation and the complex alignment procedure of retinal identification, including focusing on the retina and maintaining alignment with the direction of gaze of the subject. It would also be advantageous to have such an identification technique which could be used with minimal cooperation of the subject.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that the iris and pupil of the eye, especially the human eye, may be used for identification. Therefore, the present invention provides an identification technique based upon the recognition of the unique features of the iris and pupil, referred to herein as "iris identification".

Iris identification shares the advantages of retinal identification, but can be performed more easily because the iris and pupil are more readily visible. In addition, some of the features of the iris and pupil are highly regular, providing a geometric pattern which facilitates automatic identification. An important feature of the iris, however, is that its visible features change substantially as the pupil of the eye expands or contracts. Therefore, an important aspect of effective iris identification is to provide a technique which takes into account these changing features.

The method of identification according to the invention includes illuminating an eye, obtaining an image of the iris and pupil of the eye, and comparing the obtained image with stored image information to identify the eye. Illuminating the eye may include driving the pupil of the eye to a predetermined size, which may be done by directing light onto the retina of the eye and adjusting the intensity of the light until the pupil reaches the predetermined size. The pupil may be driven to a plurality of predetermined sizes, and a respective image of the iris and the pupil may be obtained at each of the predetermined sizes. The image obtained at each predetermined pupil size may be compared with stored image information from an eye with the same pupil size.

In another embodiment, illuminating the eye includes obliquely illuminating the iris to provide shadows of elevation-dependent features. The iris may be illuminated by a plurality of spatially separated, relatively monochromatic light sources, so that each shadow of one of the elevation-dependent features will lack a color corresponding to one of the light sources.

The comparing of the obtained image with stored image information may be a point-by-point comparison with a stored reference image. In an alternative form of comparison, a set of descriptors may be derived from the just-obtained image and these descriptors may be compared with reference descriptors derived from a previous image.

The system according to the invention includes means for illuminating an eye, means for obtaining an image of the iris and pupil of the eye, and means for comparing the obtained image with stored image information to make an identification. The illuminating means may include an adjustable light source for illuminating the eye at a selected value. In addition, the illuminating means may include control means which receive the obtained image of the eye and detect the size of the pupil, the control means being operable for adjusting the adjustable light source to a selected light intensity for controlling the size of the pupil. The control means may further be operable to control the size of the pupil to obtain a plurality of predetermined pupil sizes.

The illuminating means may also include a plurality of relatively monochromatic, spatially separated light sources for providing shadows of elevation-dependent features. Each resulting shadow of an elevation-dependent feature will lack a color corresponding to the color of one of the light sources.

The image-obtaining means may include a camera, and the comparing means may include a processor responsive to the output from the camera. The processor may include storage means for storing reference descriptors derived from a previous image, so that the processor may be operable to derive a set of descriptors from the just-obtained image and to compare these descriptors with the reference descriptors stored in the storage means.

Other objects, features and advantages of the invention will be apparent from the following description, together with the accompanying drawings and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic view of the iris and pupil of an eye, showing the major visible features.

FIG. 2 is a general flow chart showing the major functions performed in the method of the invention.

FIG. 3 is a simplified block diagram showing the flow of information through a system according to the invention.

FIG. 4 is a flow chart showing in greater detail an embodiment of the method of FIG. 2.

FIG. 5 is a flow chart showing a method of measuring rate of pupil size change according to the invention.

FIG. 6 is a simplified flow chart showing a method of side illumination according to the invention.

FIG. 7 is a general flow chart showing a method of comparing according to the invention.

FIG. 8 is a schematic diagram showing the electrical and optical characteristics of a system according to the invention.

FIG. 9 is a plan view of a viewing screen for use in the system of FIG. 8.

FIG. 10 is a functional block diagram of one embodiment of the system of FIG. 3.

FIG. 11 is a diagram illustrating the illumination control circuit of FIG. 10.

FIG. 12 is a flow chart of the operation of the processor of FIG. 10 according to the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. General Description

FIG. 1 is a simplified drawing of an eye 10, showing in detail the features of the iris 20 and pupil 30. Eye 10, as shown, is a human eye, but some other animals have a similar eye structure, permitting identification by the method of the invention. As shown, pupil 30 is a black, light receptive orifice, while iris 20 may have any of a broad range of hues and may be irregularly pigmented.

The iris 20 and pupil 30 are closely related in function. More precisely, one function of iris 20 is to control the size of pupil 30. Therefore, in addition to the visible features of iris 20 relating to pigmentation, a number of visible features relate to the movements made by iris 20 in order to contract and dilate pupil 30. In general, iris 20 may be divided into the ciliary area 22, which is an annular region at its periphery, and the pupillary area 24, which is an annular area bordering pupil 30. In general, the tissue of iris 20 is soft and loosely woven, and the illumination which enters the pupil 30 and falls on the retina of eye 10 controls muscles in iris 20 causing the size of pupil 30 to grow larger or smaller to regulate the amount of light entering pupil 30. The change in the size of pupil 30 results from involuntary reflexes and is not under conscious control.

Among the pigment-related features of iris 20 are pigment spots 26, some types of which are known as moles, freckles, or nevi, and crypts 28. Pigment spots 26 are random concentrations of pigment-bearing cells in the visible surface of the iris 20, and generally appear in the ciliary area 22. The color of the pigment spots 26 may be nearly black—darker than the darkest brown iris 20. In general, pigment spots 26 are quite stable in number, position and color. Crypts 28, unlike pigment spots 26, relate both to pigmentation and to the surface structure of the iris 20. Iris 20 includes an anterior layer which is directly visible and a posterior layer behind it, which is very darkly pigmented. The anterior layer ranges in pigment from a very light, almost white color to a dark brown color. If the anterior layer is very light or white, the iris 20 appears blue due to the pigment in the posterior layer, and if iris 20 is relatively light in color, the areas in which it is relatively thin will appear somewhat darker, due to the dark color of the posterior layer. These darkened areas may occur, for example, in the features referred to as crypts 28, which resemble sharply demarcated excavations and are typically located adjacent to the collarette 32, the boundary between ciliary area 22 and pupillary area 24.

In addition to the pigment-related features of the iris 20, several other visible features relate to its function of controlling the size of pupil 30. Collarette 32, discussed briefly above, is the boundary between the ciliary area 22 and the pupillary area 24, and is typicallly a sinuous line which forms an elevated ridge running roughly parallel with the margin of the pupil 30. The collarette 32 is the thickest part of iris 20.

Extending radially in relation to the center of pupil 30 are radial furrows 34. A typical radial furrow 34 may begin near pupil 30 and extend through collarette 32. Radial furrows 34 are creases in the anterior layer of iris 20, from which loose tissue may bulge outward, and it is this loose tissue which permits the iris 20 to expand or contract, changing the size of pupil 30. The positions of radial furrows 34 in relation to each other are stable.

Similarly, concentric furrows 36 are creases from which loose tissue may bulge outward, but their shape is generally circular, concentric with the pupil, so that they permit expansion and contraction of the iris in a different direction than radial furrows 34. Concentric furrows 36 typically appear in the ciliary area 22, near the periphery of iris 20.

Eye 10 may also have any of several rare anomalous visible features (not shown) unrelated to pigment or to controlling the size of pupil 30. Due to aging or trauma, atrophic areas may appear on iris 20, resulting in a "moth-eaten" texture. Tumors may grow on the iris. Congenital filaments may occur connecting the iris to the lens of the eye.

Finally, the most striking visible feature of eye 10 is typically pupil 30. As shown in FIG. 1, pupil 30 may not be exactly circular in shape, and its deviation from a circle is a visible characteristic. At the margin of pupil 30 is a protruding portion of the posterior layer of iris 20, the pigment frill 38. Pigment frill 38 typically has a very dark brown color.

The present invention, as discussed above, is based on the discovery that the iris of the eye may be used for identification. This discovery involves both the uniqueness of the iris and the stability of the iris over time. It has been discovered that every iris is unique, particularly in the detailed structure of the front or anterior layer. Not only are the irises of the eyes of identical twins different, but the iris of each eye of any person is different from that of his other eye. Furthermore, although specific details of the appearance of an iris vary dramatically, depending on level and direction of illumination, the basic, significant features of the iris remain extremely stable and do not change over a period of many years. Even features which do develop over time, such as the atrophic areas discussed above, usually develop rather slowly, so that an updated iris image will permit identification for a substantial length of time. A sudden or rapid change in such a feature may result in a failure to identify an individual, but this may alert the individual to the possibility of pathology of the eye. In general, the visible features of the iris and pupil may be used to identify an eye.

The method of the invention may be generally understood from FIG. 2, a flow chart showing the basic functions performed in identifying an eye from the visible features of the iris and pupil. As shown in block 40, the first function is to illuminate the eye. Because the visible features of iris 20 and pupil 30 vary depending on illumination, the illumination of the eye will be performed to obtain a desired set of features. Then, in block 50, an image of iris 20 and pupil 30 is obtained, including the desired features. In block 60, this image is compared

with stored image information in order to identify the eye.

FIG. 3 is a block diagram showing the major functional components of the system according to the invention. Illuminating means 70 provides light having desired characteristics to the eye 10 under observation. Eye 10 responds to the illumination provided, so that iris 20 and pupil 30 take a characteristic shape. In addition, iris 20 reflects light to imaging means 80, which obtains an image of iris 20 and pupil 30. This image is then compared with stored image information by comparing means 90 in order to identify the eye.

From these basic principles, a variety of methods and systems may be provided for identifying an eye from the iris and pupil.

II. Methods of Identification

A number of methods for implementing the invention are shown in FIGS. 4–7. Each of these figures is a flow chart showing a sequence of steps performed in a process of identifying an eye from the iris and pupil.

FIG. 4 shows a basic method of performing the invention in which the pupil is driven to at least one predetermined size at which an image is taken. In step 42, the pupil is illuminated at one extreme of the range of illumination, either an extreme of darkness or of brightness. The extreme may be determined by the capabilities of the system or may be arrived at by a straightforward series of steps. For example, the illumination may gradually be increased from a moderate level of illumination to an illumination beyond which further illumination does not decrease the size of the pupil. A more efficient approach, however, is to drive the pupil until its size is larger or smaller than the largest or smallest of the predetermined sizes at which an image will be obtained. From this extreme illumination, it will then be possible to drive the pupil to the predetermined size for imaging.

From the size which it has after step 42, the pupil is driven to the first predetermined size in step 44. Assuming that the extreme of illumination was darkness, the illumination will be increased in step 44 until the pupil contracts to a predetermined size. When the pupil reaches this size, an image is obtained and stored in step 52 for subsequent comparison. Then, in step 54, a test is run to determine whether the number n, a count of the images obtained, is greater than or equal to the number N of predetermined sizes at which images are to be obtained. If not, the count of the images obtained, n, is incremented in step 46, and steps 44, 52 and 54 are repeated for the next predetermined size. When all of the predetermined sizes have been imaged, step 54 will determine that n is equal to N, and image comparison will be done in step 62. In step 62, the image at each of the N predetermined sizes is compared with stored image information obtained from an eye illuminated to have the same predetermined pupil size.

Many variations may be made in the basic method of FIG. 4. For example, the largest size which the pupil attains as illumination is decreased could be treated as the first predetermined size, so that an image would be obtained after step 42. If the maximum size of the pupil remains relatively constant over time, this size will be valuable as a descriptor of the eye.

FIG. 5 illustrates another variation which can be made in the basic method of the invention. This variation includes measuring the rate at which the pupil size changes between two predetermined sizes. In step 142, light from a light source is directed onto the retina of the eye in order to control the size of the pupil through reflex action. Then, in step 144, the position or intensity of the light source is adjusted in order to obtain the first predetermined pupil size. In other words, as an alternative to varying the light intensity, the light source may be moved from a central position in which it directs light onto the central area of the retina toward an oblique position in which it directs light at an angle through the pupil onto the more peripheral parts of the retina, or vice versa. After each adjustment, in step 144, a test is performed in step 146 to determine whether the first predetermined size has been obtained. If not, the adjustment is again performed in step 144, and this loop is repeated until the first predetermined size is obtained.

After the first predetermined size is obtained, the illumination of the retina is changed at a predetermined rate in step 148. The changing of the illumination is continued until the test in step 150 determines that a second predetermined size has been attained. At that point, the time necessary to go from the first predetermined size to the second predetermined size at the predetermined rate will provide a measure of the rate of change, in step 152. This measure could also be related to the change in size between the first and second predetermined size and to the specific predetermined rate at which the illumination is changed in step 148. The resulting value may then be used to identify the eye. It should be borne in mind, however, that the rate of change of pupil size may vary for individuals whose reflexes are affected by emotion, drugs, and so forth, so that the rate of change of pupil diameter should generally provide only a secondary indication of identity.

The methods shown in FIGS. 4 and 5 could be combined in a simple manner by making minor modifications to the steps performed in FIG. 4. Step 44 could first be performed to obtain the first predetermined size, corresponding to steps 144 and 146 in FIG. 5. After the performance of steps 52, 54 and 46, step 44 could again be performed by changing the illumination at the predetermined rate to obtain the second predetermined size, corresponding to steps 148 and 150 in FIG. 5. Then, in addition to obtaining the image in step 52, the rate of change of pupil size could also be determined in step 152.

FIG. 6 illustrates a routine which may similarly be included in step 52 of FIG. 4, to obtain an image of the iris of an eye in which the elevation-dependent features are shown with greater contrast. This is done by illuminating the iris from a plurality of oblique positions. The illumination from each position is provided by a relatively monochromatic light source to produce shadows of elevation-dependent features lacking the color of that light source. In step 250, illumination from position m is performed using the color m of the corresponding relatively monochromatic light source. This illumination will produce shadows lacking the color m, and an image of these shadows is obtained in step 252. Then, in step 254, a test is performed to determine whether m has reached M, the total number of positions from which the iris is to be illuminated. If not, m is incremented in step 256, and the loop including steps 250, 252 and 254 is repeated until m equals M. When m reaches M, the shadow images may be processed in step 258 to prepare them for subsequent comparison with reference images.

The method of FIG. 6 is especially beneficial for obtaining images of elevation-dependent features such as radial furrows 34. As can be seen from FIG. 1, however, radial furrows 34 may extend radially in any direc-

tion, so that illumination from any one direction will provide an excellent shadow image of radial furrows **34** which extend perpendicular to that direction of illumination. Radial furrows which extend parallel to the direction of illumination, however, will produce poor shadow images. Therefore, to produce the best images of radial furrows **34**, it is desirable to obtain a number of shadow images and to combine them in processing step **258**.

The comparison of images having the same pupil sizes in step **62** of FIG. **4** may be performed in many ways. Onr straightforward method would be to store the reference image as a pixel-by-pixel image resulting from a scanning camera such as a conventional video camera. The image obtained would then be obtained with a similar video camersa, as discussed below in relation to FIG. **8**, to obtain a pixel-by-pixel image of the eye. The two images could then be compared, pixel-by-pixel, to determine whether they were the same image. The comparison could be made in a way which would take into account differences in the overall intensity of the two images. This method of comparison, although simple to implement, is extremely sensitive to registration. In other words, the test would only result in a conclusion that the two images were of the same eye if the eyes shown in the two images were of the same size and were positioned identically. Therefore, it will be preferable to perform a comparison algorithm which is not so sensitive to registration.

One comparison algorithm which would not be so sensitive to registration is the correlation algorithm, which has many variations. The effect of an offset in the positioning of the two images could be eliminated based on an algorithm for determining the center of gravity of the pupil in each image. These algorithms could thus be combined to reduce registration sensitivity.

Another comparison method which would not be highly sensitive to registration would be a method using optical signal processing. Hecht, J., "Light Modulators Help Crunch Image Data", *High Technology*, Vol. 5, No. 1 (January, 1985), p. 69–70, 72, discusses techniques for optical comparison which would be readily applicable to the present invention.

FIG. 7 illustrates another method of comparison which could be used in the present invention. In step **64**, descriptors are extracted from the image obtained, which may be the image obtained in step **52** of FIG. **4**. One example of descriptors which could be extracted would be the angular positions and lengths of the radial furrows **34**. Another set of descriptors would be the shape of the pupil **30** or of the pigment frill **38**. Other descriptor sets could be obtained relating to the positions and sizes of pigment spots **26**, crypts **28** and anomalous features such as atrophic areas, tumors and congenital filaments. Similarly, generally circular features such as collarettes **32** and concentric furrows **36** could be described respective descriptors. All of these descriptors would then be compared with the descriptors of a reference image in step **66**. Because several features of the iris **20** and pupil **30** lend themselves readily to mathematical description, this method of comparison should be effective and should not be sensitive to registration. A number of algorithms for extracting descriptors are discussed below.

Many types of equipment could be used to implement the invention and to perform the methods set forth above. One example of circuitry for performing these functions is described below.

III. Identification Systems

As noted above, the necessary equipment for performing iris recognition according to the invention could take many forms. The major functional components of any such system, however, will be those shown in FIG. **3**, including illuminating means **70**, imaging means **80** and comparing means **90**.

FIG. **8** shows a system for illuminating and obtaining an image of the eye. The system includes a simplified electrical circuit for controlling illumination and an optical arrangement in which the image of fixation target **71** seen by eye **10** and the reflected image of iris **20** and pupil **30** to camera **84** are approximately coaxial. It is presently preferred to provide a target image, upon which eye **10** may be fixated, concentric with visual axis **16** for reference purposes. It is also preferred for camera **84** to capture the reflected image concentric or nearly concentric with visual axis **16**, because if the image were obtained far from the axis **16**, it would be distorted, which would then have to be compensated by appropriate processing. Additional illumination of iris **20** is provided obliquely to create good shadows of elevation-dependent features.

In the system of FIG. **8**, the source of light is a filament *72a* which receives power from power source *72b* connected in series through variable resistor *72c*, which may be an electrically controlled potentiometer. The light emitted by filament *72a* is collimated by lens **73** and illuminates target **71**, which may have any appropriate shape.

The image of target **71** is then focused by movable lens **74** and transmitted through monitor beam-splitter **76** onto axial beam-splitting plane **82**. The image is then reflected from the central mirrored area *82a* through pupil **30** onto retina **12**.

The optical system shown in FIG. **8** includes means for placing target **71** at any desired optical distance from eye **10**. Target **71** can thus be made visible to subjects with a wide variety of refractive states. The refraction or bending of light rays at the air interface of cornea **14**, for example, is substantial and varies for different subjects. The necessary adjustment of optical distance is obtained by moving lens **74** toward or away from target **71** until target **71** is optically conjugate with retina **12**. If necessary to compensate for astigmatism, lens **74** may also be a variable sphero-cylindrical lens system of any of the well-known types. Target **71** will be optically conjugate with retina **12** when, as shown in FIG. **8**, the light from each point on target **71** comes to a focus at a corresponding point on retina **12**. The convergence or divergence of light from target **71** due to the adjustment of lens **74** will thus compensate for the optics of eye **10**.

Oblique light sources *78a* and *78b* provide illumination to the iris **20** and may be controlled similarly to filament *72a* by using a variable resistor (not shown). The resulting reflection will depend on the positioning of oblique light sources *78a* and *78b*, because the iris is a diffuse reflector, reflecting light according to a cosine law in which the amount of reflected light in the direction normal to the iris varies as the cosine of the incident angle of the light.

Axial beam-splitting plane **82** serves the two functions of directing the image of target **71** from lens **74** toward eye **10** and also transmitting the reflected image of iris **20** to camera **84**. In the arrangement shown in FIG. **8**, which is not to scale, beam splitting plane **82** is

a thin transparent sheet such as glass on which mirror **82***a* is formed for reflecting the image. Mirror **82***a* may be a microscopically thin, reflective film or coating, such as a metal, on the central part of plane **82** and on the side toward eye **10**, to provide a first surface mirror. It may, for example, have an elliptical shape. The reflected image of target **71** is directed through pupil **30** onto the retina **12** of eye **10** to control the size of pupil **30**. The reflected light from iris **20**, which will emerge from eye **10** quite divergently, may pass through the clear, transparent area of beam splitting plane **82** which surrounds mirror **82***a*. Mirror **82***a*, however, will create a non-transparent area in the center of plane **82**, preventing an intense reflection of the target image from cornea **14** from reaching camera **84**. Since the reflected target image may be the brightest object in the field of view of camera **84**, this may significantly reduce the noise in the image of iris **20** and pupil **30**. Monitor beam-splitter **76** will provide an image of the intense reflection from cornea **14**, as reflected by mirror **82***a*, and this reflection image may be used to position the eye according to known eye-positioning techniques. Proper positioning of the eye will help to provide a standardized image of iris **20** and pupil **30** and will ensure that the intense reflection of the target image does not reach camera **84**.

FIG. **9** shows viewing screen **86** of camera **84**, upon which are marked peripheral points **88***a* and central point **88***b*. Peripheral points **88***a* may be used to measure the size of pupil **30**, as shown. When the perimeter of pupil **30** touches each of the peripheral points **88***a*, pupil **30** has been driven to a predetermined size at which an image should be obtained. Central point **88***b* may be useful in aligning pupil **30** so that size may be properly tested. As shown in FIG. **9**, a grid may also be provided on viewing screen **86** to assist in the determination of the size of pupil **30** when it does not meet peripheral points **88***a*. Alternatively, concentric markings could be provided on screen **86**, as disclosed in U.S. Pat. No. 3,966,310, issued to Larson.

It will be apparent from FIGS. **8** and **9** that the distance between camera **84** and eye **10** must be fixed in order to provide a standardized image. This can be done by providing a headrest (not shown) against which the forehead above eye **10** may be positioned. The headrest may also be equipped with appropriate additional surfaces for darkening a space around eye **10**. This permits the system to provide a condition of nearly zero illumination to retina **12**.

The system shown in FIGS. **8** and **9** may be manually operated to obtain the predetermined pupil size touching peripheral points **88***a*. First, variable resistor **72***c* is manually adjusted to its maixmum resistance, effectively turning off filament **72***a*. Oblique light sources **78***a* and **78***b* are activated to provide a desired level of illumination upon iris **20**. Then, variable resistor **72***c* is gradually turned to a lower resistance until the image of target **71** begins to illuminate retina **12**. The operator, looking at viewing screen **86**, will see the pupil beginning to decrease in size as the illumination from filament **72***a* increases. When the perimeter of pupil **30**, as seen through viewing screen **86**, just touches peripheral points **88***a*, the operator may activate camera **84** to obtain an image of iris **20** and pupil **30**. If further, predetermined sizes are marked by points in viewing screen **86**, the operator may continue to adjust variable resistor **72***c* until those additional predetermined sizes of pupil **30** are shown in viewing screen **86** and are imaged when camera **84** is activated.

The optics of FIG. **8** cold be modified in many ways within the scope of the invention. For example, rather than providing a transparent beam-splitting plane **82** with a centered mirror **82***a*, the beam-splitting plane could be defined by two prisms positioned against each other or by a half-silvered mirror or other partially reflecting surface. Such arrangements, however, will result in a greater loss of intensity of the collimated light and of the reflected image than the arrangement of FIG. **8**. If such an arrangement is desirable, therefore, it may be appropriate to provide additional illumination of the iris **20**, as discussed below in relation to FIG. **11**.

FIG. **10** shows a block diagram of electrical components performing the functions set forth in FIG. **3**. Iris recognition system **100** in FIG. **10** includes illumination control circuit **170**, camera **180** and processor **190**. Illumination control circuit **170** and camera **180** operate under the control of processor **190**. In response to control signals, illumination control circuit **170** illuminates eye **10** both to control the size of pupil **30** and to provide a reflected image of iris **20** and pupil **30**. This reflected image is received by camera **180**, which may be a video camera which converts the reflected image into an image signal for processor **190**. Processor **190** then compares the image signal with a reference image in order to identify the human being.

FIG. **11** shows in greater detail the illumination system of iris recognition system **100**. As shown in FIG. **11**, illumination control circuit **170** controls light sources **172**, **174**, **176** and **178**. In general, these light sources should be as small as possible to minimize noise in the reflected image from eye **10**. Light source **172**, which illuminates retina **12**, as discussed in relation to FIG. **8**, may do so through a fixation target **71** and a beam-splitting plane **82**. Light source **172** will thus provide a target image upon which eye **10** will fix and focus, thereby becoming aligned for imaging. The target image should preferably be a distant object to discourage accommodation which would reduce pupil size. Rays from the target image may be relatively tightly collimated and subtend a small angle in order to provide a more critical, centralized alignment, or may be less tightly collimated if additional iris illumination is desired. The target image could also be compound, such as cross hairs in one plane which must be aligned on a designated part of a background at another plane to obtain alignment of the pupil, although this would depend on the acuity of the eye **10**. Alternatively, a polarizing ring-sight device could be used to create concentric circles on the retina independent of the refraction of eye **10**. The target image should not be extremely bright, because that would also reduce the size of pupil **30**.

As mentioned above, the target image may be used to cause the subject to align pupil **30** for optimal imaging. In addition, however, it would be possible to provide an automatic alignment system using a feedback circuit and a servo-control mechanism to adjust the relative positions of the iris **20** and the camera **180**. This adjustment of position could be accomplished with rotatable mirrors, plano-parallel plates, or prismatic devices such as Risley prisms. Alternatively, the head rest (not shown) can be moved in order to orient the head.

In addition to pupil-driving light source **172**, the illumination system shown in FIG. **11** includes oblique light sources **174**, **176** and **178**. As discussed above in

relation to FIG. 6, each of these oblique light sources **174**, **176** and **178** may provide a relatively monochromatic beam of light, so that the shadows resulting from the oblique positioning of these light sources will lack the corresponding color, facilitating the subsequent processing of the shadow images. Each source **174**, **176** and **178** could be a relatively monochromatic source such as an LED or a solid state laser.

Although FIG. **11** shows an arrangement in which three light sources are provided at approximately 120° angles from each other, the number of light sources and their relative angular positions could be varied in many ways. In addition, other arrangements could be used to obtain additional information about elevation-dependent features of iris **20**. For example, more than one camera **180** could be provided, or a single camera **180** could be provided which could be moved between a plurality of positions in order to obtain three-dimensional information. Any other means for obtaining an image could be used, including laser-based techniques such as holography and including flying spot scanning by mechanical or electrooptical techniques.

FIG. **12** shows a flow chart of the operation of processor **190** in FIG. **10**. Processor **190** will at first be initialized in step **342**, in order to provide appropriate memory contents and in order to perform the necessary loading of programs. In addition, as a part of initialization, processor **190** may provide control signals causing camera **180** to begin to operate and providing power to illumination control circuit **170**. In step **344**, processor **190** provides control signals to illumination control circuit **170** causing pupil-driving light source **172** to illuminate the retina **12** at the extreme illumination of the range available. In addition, oblique light sources **174**, **176** and **178** may be activated to provide sufficient illumination so that camera **180** is able to provide an image of pupil **30** to processor **190**.

When the extreme size of the pupil has been obtained in step **344**, the pupil is then driven across the range of its size in step **346**. This is performed by gradually increasing the illumination from pupil-driving light source **172**. After each increase in intensity, the pupil size is measured in step **348** and tested in step **352** to determine whether it equals the desired predetermined size x_n. For the first iteration, the first predetermined size x_1 will be obtained after the necessary reiteration of the loop including steps **346**, **348** and **352**. Oblique light sources **374**, **376** and **378** will also be activated, either at the same time or in sequence according to a method like that shown in FIG. **6**, in order to obtain an image of the iris **20** and pupil **30** of eye **10**. Oblique light sources **374**, **376** and **378** should be activated in a way which minimizes the constriction of pupil **30**. Therefore, it may be necessary to activate them before or while the illumination from light source **172** is increased.

From this image, processor **190** will then extract the nth descriptor set in step **362**. For the image obtained when the pupil size is x_1, the first descriptor set will be obtained. Then, in step **364**, n is tested to determine if it is equal to N, the total number of descriptor sets to be obtained. If not, n is incremented in step **366**, and the loop including steps **346**, **348**, **352**, **354**, **362** and **364** is repeated for the next value of n. This loop will be repeated until step **364** determines that n equals N.

When n equals N, the resulting descriptor sets are compared with one or more reference descriptor sets in step **368**. If the objective is to determine whether the eye **10** is that of one of a population of people, each descriptor set resulting from step **362** will be compared with the corresponding descriptor sets of the members of that population. On the other hand, if the objective is simply to confirm that an eye **10** is that of a specific individual, it is only necessary to compare the descriptor sets resulting from step **362** with the corresponding descriptor set of that individual, which may, for example, be stored on a credit card, identification card, or other identifying document, or in the memory of a computer system to which the processor **190** has access.

Upon the completion of step **368**, an appropriate indication may be provided to the operator of iris recognition system **100** that the eye **10** has been identified or has not been identified, completing the operation shown in FIG. **12**. At this point, the operator may choose to initiate further iris recognition by repeating the process shown in FIG. **12**.

Processor **190**, in extracting descriptors in step **362**, may perform a wide variety of algorithms. A number of such algorithms are set forth in Ballard, D. H. and Brown, C. M., *Computer Vision*, Prentice-Hall, Englewood Cliffs, N.J., 1982; Duda; R. O. and Hart, P. E., Pattern Recognition and Scene Analysis, Wiley, New York, 1973; and Rosenfeld, A., *Digital Picture Processing*, Academic Press, New York, 1982. Many of these algorithms use classical mathematical techniques such as difference operators, taking a differential equation of selected points and testing a rate of change, and mask matching or surface fitting, fitting to a known shape at various orientations. Although these and other such algorithms are known, they have not previously been applied to the specific features of the iris and pupil.

The location, size and deviation from circularity of the pupil could provide a number of descriptors. The pupil could first be detected using a boundary detection algorithm or an edge detection algorithm, which may, for example, detect an abrupt gray level change. Systems performing pupil size measuring algorithms are disclosed, for example, in U.S. Pat. No. 3,598,107, issued to Ishikawa et al, and U.S. Pat. No. 3,533,683, issued to Stark et al. A Hough transform algorithm for detecting circles, which maps curves into the transform space according to characteristics such as curvature, could be used on a boundary or edge which is detected. Alternatively, the received image could be subjected to a thresholding algorithm after which a region-growing or aggregation algorithm, such as the blob coloring algorithm discussed by Ballard and Brown, could be performed to find the largest connected region of pixels with intensity values below threshold. Since the pupil is central, the region could be grown outward from a central dark pixel in the image, progressively aggregating the adjacent dark pixels until the pupil boundary is reached, beyond which adjacent pixels will not be dark. This will provide a measure both of pupil size and of location, as the center of the pupil may be determined from its boundary. This may be done, for example, by taking slices through the dark region in a number of radial directions to select a largest diameter. To eliminate the chance that the largest diameter is not the true diameter, as, for example, if it extended to a notch or other irregularity in the boundary, the least sum of squares from its center to the boundary could be taken and compared with that of the second largest diameter. The center and radius giving the least sum of squares would define the circle most closely approximating the pupil boundary, and the residual sum of squares would be a quantitative measure of deviation of the pupil from

circularity, a "figure of merit" for the pupil. This figure of merit would be a useful descriptor of the pupil.

The characteristics of the radial furrows could also be used to obtain descriptors. From the center of the pupil, the precision of radiality of the radial furrows can be obtained. Line detection algorithms and streak or thick line detection algorithms could be used to locate the radial furrows. The radial furrows may then be compared with true straight lines radiating from the center by obtaining a sum of squares of differences, indicating radiality. Some furrows, rather than being entirely straight, include, for example, two offset parts, each of which is generally radial, the two being connected by a non-radial jog. A measure of thickness could be obtained for such a furrow by determining how wide a streak is necessary to cover the furrow. Also, the Hough transform algorithm for detecting straight lines could be applied to obtain such descriptors as the length, angular location and direction of radial furrows.

The concentric furrows may be detected and described using edge detection algorithms and the Hough transform algorithms for detecting curved lines. Similarly, the collarette and the contours of the crypts along it could be described using edge detection algorithms and chain coding algorithms. In addition, points of high curvature along these contours could be located and represented for matching purposes, which may be more efficient. Isolated crypts could be described by a thresholding algorithm, followed by a region-growing algorithm and then algorithms obtaining the center of gravity and the lower order central moments, as described by Duda and Hart.

Spot detection algorithms, detecting high contrast spikes in areas of relatively constant gray level, could detect small pigment spots. More generally, algorithms like those for measuring the pupil could be used to obtain location and lower-order central moments of pigment spots, and other such features. Also, high-curvature points along the contour of a feature could be detected and described.

Finally, the color of the iris generally could be found by an algorithm obtaining a histogram in a three-dimensional RGB (Red-Green-Blue) color space, as described by Ballard and Brown at page 153. The peak in the histogram will provide a descriptor of color.

As noted above, the embodiment of FIGS. **8–12** could be modified in many ways within the scope of the invention. Processor **190** may control the operation of illumination control circuit **170** and camera **180** in any appropriate manner to obtain images for processing. For example, the image from camera **180** may be constantly received and processed by processor **190** to determine the appropriate level of illumination by illumination control circuit **170**. At the same time, when it is desired to obtain an image at one of the predetermined sizes of pupil **30**, processor **190** may control illumination means **170** to maintain a temporarily constant level of illumination while camera **180** obtains a complete image for processing.

Although the present invention has been described in connection with a plurality of preferred embodiments thereof, many other variations and modifications will now become apparent to those skilled in the art. It is preferred, therefore, that the present invention be limited not by the specific disclosure herein, but only by the appended claims.

What is claimed is:

1. A method of identification of a person, comprising:

storing image information of at least a portion of the iris and pupil of the person's eye;

illuminating an eye, of an unidentified person having an iris and a pupil;

obtaining at least one image of at least the same portion of the iris and pupil of the eye of the unidentified person; and

comparing at least the iris portion of the obtained image with the stored image information to identify the unidentified person.

2. The method of claim **1** in which illuminating comprises driving the pupil of the eye to at least one predetermined size, comparing at least the iris portion of the obtained image with stored image information obtained from an eye with its pupil having the same predetermined size.

3. The method of claim **2** in which driving the pupil comprises directing light onto the retina of the eye and adjusting the intensity of the light until the pupil reaches the predetermined size.

4. The method of claim **2** in which driving the pupil comprises driving the pupil to a plurality of the predetermined sizes; the step of obtaining at least one image comprising obtaining a respective image at each of the predetermined sizes of the pupil.

5. The method of claim **4** in which illuminating comprises changing the intensity of illumination at a predetermined rate between at least two of the predetermined pupil sizes and measuring a rate of change of pupil size as the illumination intensity changes.

6. The method of claim **1** in which illuminating comprises illuminating the iris obliquely for providing shadows of elevation-dependent features.

7. The method of claim **6** in which illuminating the iris obliquely comprises illuminating the iris from a plurality of spatially separated light sources for providing shadows of elevation dependent features extending in a plurality of directions.

8. The method of claim **7** in which illuminating the iris from a plurality of spatially separated light sources comprises illuminating the iris from a plurality of relatively monochromatic light sources, each light source providing shadows lacking a corresponding color of light.

9. The method of claim **1** in which comparing the obtained image with the stored image information comprises comparing at least the iris portion of the obtained image with a stored reference image for identifying the person.

10. The method of claim **1** in which comparing the obtained image with stored image information comprises deriving a set of descriptors of at least the iris portion of the obtained image and comparing the derived descriptors with stored reference descriptors derived from a previous image for identifying the person.

11. The method of claim **10** in which deriving a set of descriptors comprises deriving descriptors of the pupil.

12. The method of claim **10** in which deriving a set of descriptors comprises deriving descriptors of a pigment frill around the pupil.

13. The method of claim **10** in which deriving a set of descriptors comprises deriving descriptors of a collarette in the iris.

14. The method of claim **10** in which deriving a set of descriptors comprises deriving descriptors of at least one concentric furrow in the iris.

15. The method of claim 10 in which deriving a set of descriptors comprises deriving descriptors of at least one radial furrow in the iris.

16. The method of claim 10 in which deriving a set of descriptors comprises deriving descriptors of at least one crypt in the iris.

17. The method of claim 10 in which deriving a set of descriptors comprises deriving descriptors of at least one pigment spot in the iris.

18. The method of claim 10 in which deriving a set of descriptors comprises deriving descriptors of at least one atrophic area in the iris.

19. The method of claim 10 in which deriving a set of descriptors comprises deriving descriptors of at least one tumor in the iris.

20. The method of claim 10 in which deriving a set of descriptors comprises deriving descriptors of at least one congenital filament in the iris.

21. A system of identification, comprising:

means for storing an image of at least a portion of the iris and pupil of a person's eye;

means for illuminating an eye having an iris and a pupil;

means for obtaining an image of at least the same portion of the iris and pupil of the eye; and

means for comparing at least the iris portion of the obtained image with the stored image information to identify the person.

22. The system of claim 21 in which the means for illuminating comprises an adjustable light source for illuminating the eye at a selected value in a range of light intensity.

23. The system of claim 22 in which the means for illuminating further comprises control means responsive to at least the iris portion of the obtained image of the eye for detecting the size of the pupil and operable for adjusting the adjustable light source to a selected light intensity for controlling the size of the pupil.

24. The system of claim 23 in which the control means is further operable for controlling the size of the pupil to obtain a plurality of predetermined pupil sizes; the control means being further operable for adjusting the light source at a predetermined rate across the range of light intensity between at least two of the predetermined pupil sizes for measuring the rate of change of pupil sizes as the light intensity is adjusted.

25. The system of claim 21 in which the illuminating means further comprises a plurality of spatially separated oblique light sources for illuminating the iris obliquely.

26. The system of claim 25 in which each of the oblique light sources comprises a relatively monochromatic light source for providing shadows lacking a corresponding color of light.

27. The system of claim 26 in which each relatively monochromatic light source comprises a laser.

28. The system of claim 21 in which the image obtaining means comprises a camera, the comparing means comprising a processor responsive to an output from the camera for comparing the obtained image with stored image information.

29. The system of claim 28 in which the processor comprises storage means for storing reference descriptors derived from a previous image, the processor being operable for deriving a set of descriptors of the obtained image and for comparing the derived descriptors with the reference descriptors for identifying the person.

30. The system of claim 21 in which the image obtaining means comprises a laser.

31. The system of claim 21 further including target means for providing an object upon which the eye fixates.

32. The system of claim 31 further including focusing means for focusing the target means.

* * * * *

Related Cases Section

None.